An investigation of an infection with a protozoan parasite causing mortalities in Little Penguins (*Eudyptula minor*) on Penguin Island, Western Australia

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This thesis is presented for the degree of Master of Science of Murdoch University, 2015.

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Since at least 2001, routine post mortems of deceased penguins from Penguin Island, Western Australia, have been conducted at Murdoch University Veterinary Hospital (MUVH). In late 2011 and early 2012, a cluster of 12 cases presented with similar and characteristic gross and microscopic changes, namely birds in good body condition with hepatomegaly and splenomegaly, multifocal hepatic and splenic necrosis and numerous, small, 1-2µm diameter protozoan parasites within the necrotic foci. A review of earlier reports in the MUVH archive identified isolated similar cases from 2006 and 2008, which had been provisionally diagnosed as Avian Malaria, that is, *Plasmodium* spp. infection. An investigation was established in order to: a) definitively identify the parasite causing the mortalities, with the additional aims of: b) evaluating the live population of Penguin Island Little Penguins for the presence of parasitaemia, and c) investigating the possibility that another bird species present on Penguin Island might represent a reservoir of infection.

Ninety-four blood smears were made from 79 individual Little Penguins collected from winter to summer of 2012 and in the early spring of 2013. One smear identified intraerythrocytic organisms consistent with the blood stages of an apicomplexan parasite, for example, merozoites or early gametocytes of *Haemoproteus* or *Plasmodium*, or merozoites or sporozoites of *Babesia*. Fifty-one blood smears were made from 51 Bridled Terns (*Sterna anaethetus*) captured during two visits to the island in November 2012 and March 2013, with no parasites detected in these smears. Electron microscopy of the protozoan parasite identified it as belonging to the phylum Apicomplexa. Further identification to the level of genus was not possible.

Molecular identification of the parasite using Polymerase Chain Reaction (PCR) methodology gave inconsistent results. PCR performed by an independent laboratory identified a novel *Haemoproteus* spp. organism in 4 of 10 cases from this group; however, these results could not be replicated in our laboratory. Additional PCR using a variety of primers aimed at detecting members of the Apicomplexa identified a parasite from the family Sarcocystidae, which was subsequently identified as *Toxoplasma*. Immunohistochemistry of formalin fixed tissues also identified *Toxoplasma* in the hepatic and splenic lesions.

The distinctive mortalities which were observed in this group of penguins, and which have occurred sporadically since, appear to be attributable to a fulminant toxoplasmosis, with or without a concurrent haemoproteosis in some cases. The significance of the apparent polyparasitism in some of the birds is unknown, as the relative contribution of concurrent *Haemoproteus* infection to the lesion aetiopathogenesis cannot be quantified at this time. Though the clinical signs of infection are unknown, the gross and microscopic appearance at post mortem is sufficiently characteristic to allow a diagnosis to be made on these features. Definitive confirmation of infection may be made by immunohistochemistry or PCR.

Table of Contents

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1. Literature review

Preamble

The subject of and material for this thesis arose from observations made during routine post mortem examinations of Little Penguins (*Eudyptula minor*) conducted at Murdoch University Veterinary Hospital. The penguins, most of which were found deceased on Penguin Island as well as along the coast in the Perth and Rockingham areas, were, for the most part, identifiable as inhabitants of Penguin Island either by wingband or microchip, or by virtue of where they were found. In those birds that lacked definitive identifiers, a small number may have originated from nearby Garden Island instead.

Little Penguins

Little Penguins are the smallest species of penguin, and the only penguin native to Australia. Their distribution takes in the southern coastline of mainland Australia, the coastlines of Tasmania and the Bass Strait islands, most of New Zealand, and a number of its associated islands, including Stewart and Chatham Islands. The total population worldwide is estimated to be 500-600,000 individuals, with the largest populations on the coasts of Victoria, the Bass Strait islands and Tasmania[34,79]. According to the International Union for Conservation of Nature, the current conservation status of this species is designated Least Concern[1]. However, many individual colonies of these birds are threatened, particularly mainland colonies, and their overall numbers are considered to be in decline[2,8,34,44,79,88].

As true seabirds, Little Penguins spend extensive periods of time at sea, coming to land principally for the purposes of breeding and their annual moult. Locally, the breeding cycle sees courtship, laying and chick rearing occurring from mid-autumn to early summer, with eggs being laid from as early as April to as late as December[51]. Egg incubation, and the subsequent guarding of the chicks after hatching, requires an equal contribution from both parents. Little Penguins typically lay eggs in clutches of 2, with a 2-7 day interval between the first and the second egg, though occasionally only a single egg is produced[48,88]. Successful hatching of the eggs and rearing to fledglings depends on the capacity of a pair to provide continuous protection for the approximately 35 day incubation period[10,48], and the post-hatching guard stage, which lasts from 8 to 38 days, during which the chicks are not left alone[10,36]. The parents take turns foraging for food and attending the nest; when food is scarce, the foraging parent may be unable to gather adequate food near enough to the nest that it is able to return in time to relieve the nesting parent, and in this case hunger eventually forces the

nesting parent to abandon the eggs or chicks. The diet of the Little Penguin may include many small species of fish, squid, prawns and other small crustaceans[7,65,86], but among the local birds the bulk of their diet is made up of the sandy sprat (*Hyperlophus vittatus*), southern sea garfish (*Hyporhamphus melanochir),* blue sprat (*Spratelloides robustus*) and pilchard (*Sardinops neopilchardus, Sardinops sagax*)[50,68]. Thus, the breeding success of the penguins is closely linked to the abundance of these fish species in their local foraging grounds. And obviously, breeding success is pivotal to the ability of a colony to maintain its numbers or to replenish numbers after a population decline.

Populations of Little Penguins have been reported to be in decline from a number of locations around Australia[2,8,88]. In many cases, the causes for the population declines have been attributed to human activities, such as habitat alteration, reduction in fish stocks, pollution or entanglement in fishing paraphernalia[56,79]. In one study of 213 Little Penguins from the Otago region of New Zealand's South Island, the most commonly identified causes of death were starvation (16%); road, rail or unspecified trauma (23%); and predation by dog or ferret (23%)[38]. Since they are vulnerable to predation from introduced species, Little Penguins have abandoned or been forced from most onshore nesting sites and now nest mainly on offshore islands.

Penguin Island and threats to the population

The largest colony of Little Penguins in Western Australia breeds on Penguin Island, located roughly 40km south of the city of Perth (see Fig 2)[56]. Besides this large colony, other smaller colonies in the local area are found on Garden Island and Carnac Island a few kilometres to the north from Penguin Island. Other Western Australian colonies are found on islands near Augusta, Albany and in the Recherche Archipelago near Esperance (see Fig 1).

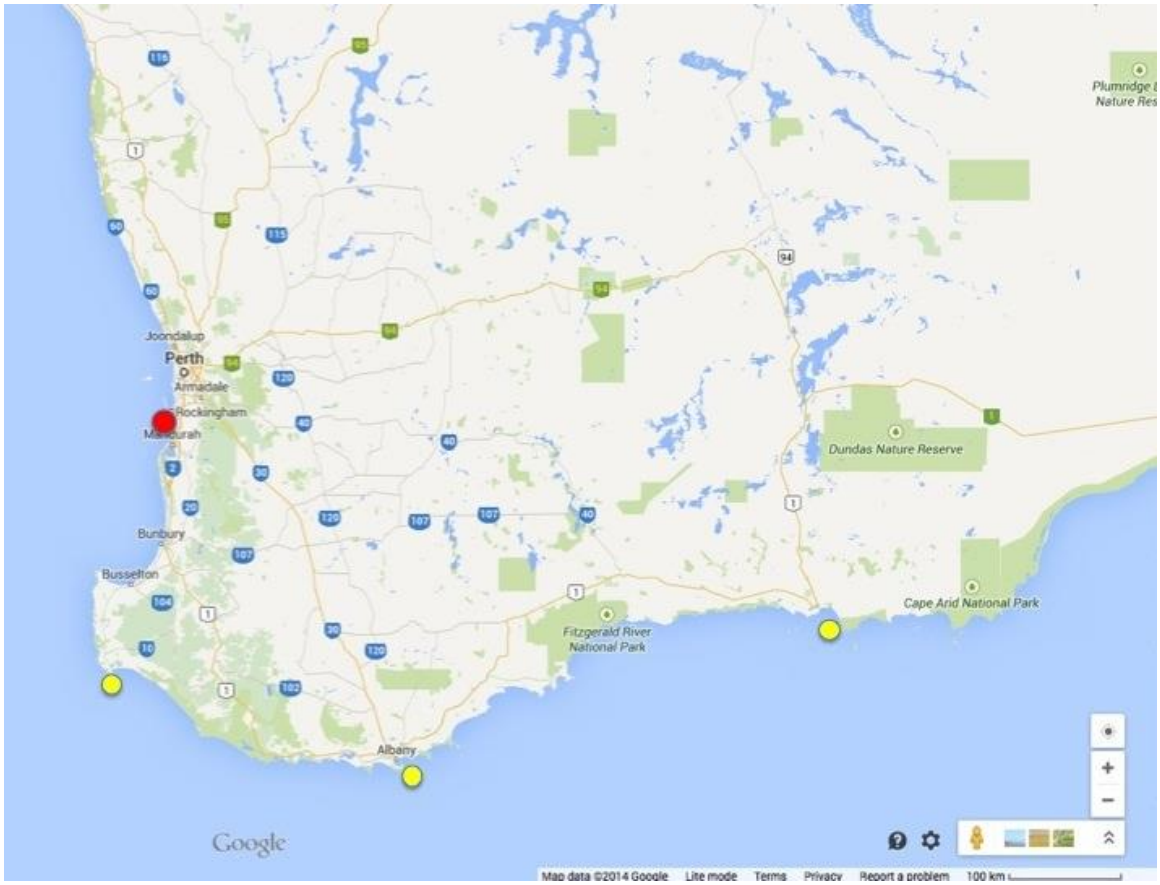


Figure 1 – Location of substantial colonies of Little Penguins in Western Australia, Penguin Island in red, other colonies in yellow

Penguin Island itself is a limestone island, one of the largest of a chain remaining after submersion of a dune ridge system now lying offshore from the City of Rockingham[62]. Many of the islands in this chain and the surrounding sea are encompassed within the Shoalwater Islands Marine Park, a conservation park, gazetted in 1990, which extends from Point Peron in the north to Warnbro Sound in the south[3]. Penguin Island is the largest of the islands within the Marine Park. The management of the marine park currently falls to the Western Australian government's Department of Parks and Wildlife, formerly the Department of Environment and Conservation, formerly the Department of Conservation and Land Management – for simplicity, these several incarnations of the responsible government agency will be referred to as 'the Department'. In the Department's current management plan, Penguin Island is designated as lying within a Special Purpose Zone (Wildlife Conservation)[3]. Though it is uninhabited, and in fact only ever had one long-term resident (Seaforth McKenzie, who lived on the island from about 1914 to 1926), Penguin Island has a long history of use as a recreation and holiday destination. Recreational use continues to this day, except during the peak breeding season of the Little Penguins, and access to the island for the community is identified as an important component of the Department's management plan. Under the terms of the plan,

the status of the colony of Little Penguins is one of several Key Performance Indicators used to gauge the efficacy of the management of the Marine Park. Specifically, the breeding success and abundance of the birds, as well as the number of entanglements of penguins in fishing equipment per year, are the stated measures by which the colony's health is quantified[3].

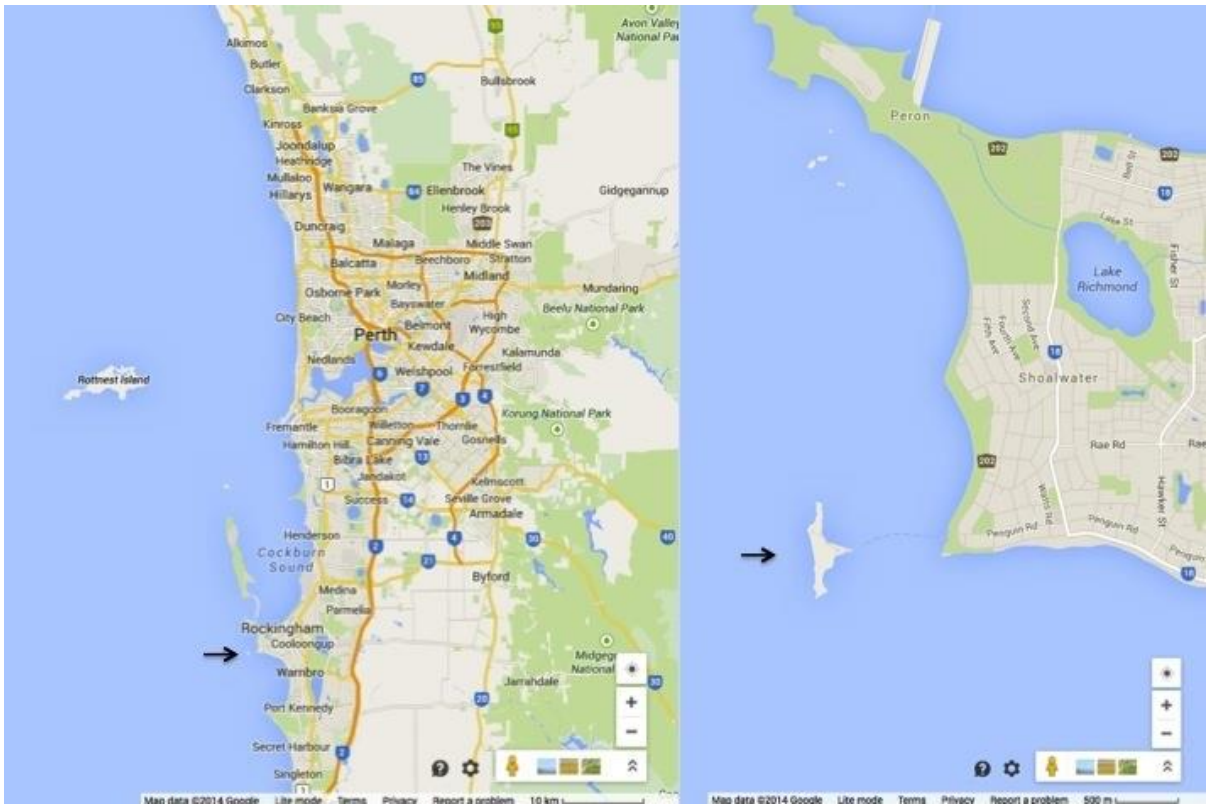


Figure 2 – Location of Penguin Island (identified by arrows) in relation to Perth and to the city of Rockingham, Western Australia

The geology of Penguin Island is not well-suited to typical Little Penguin nesting habits, consisting as it does of low shrubs growing on a sandy substrate. Here, the penguins tend not to make burrows as they do in most other colonies, since the sand is too unstable for this purpose; instead, the majority of birds nest under the low, scrubby *Tetragonia* or *Rhagodia* bushes which cover the island, with a few making nest sites from other vegetation, relatively infrequent natural structures such as limestone caves, or the remnants of human building materials and structures[49]. Since 1986, artificial nest-boxes made of plywood have been placed at various sites around the island with recorded occupancy rates ranging from 49% to 94%[49]. The good occupancy rates for the nest boxes suggest that they are at least adequate for the penguins' requirements, though there is evidence that redesign of the boxes to prevent high ambient temperatures, with the potential to induce hyperthermia, may improve their utility[75].

Naturally enough, the Little Penguins are recognised to play a key ecological role in the marine park. They are, however, notable for other reasons as well. Not only does this colony lie at the western, and near the northern, limit of the species' range[56], but the birds are also significantly larger than those from other populations[87], and this colony also has a longer breeding season than many others do. By these and other criteria, this colony is given the highest conservation status out of 256 Little Penguin colonies identified Australia-wide[18]. Given the importance of this colony, and its fortuitous location near Western Australia's largest population centre and academic institutes, there has been extensive academic and, more recently, government research and monitoring activity conducted over a relatively extended period of time[11-13,16,22,49-51,59,75,87].

In the last few decades, several population estimates have been made for different locations on Penguin Island, as well as for the whole island, using methodologies of different degrees of accuracy[87 12]. Conveniently, the penguins display a great deal of site fidelity in their choice of landing sites, reliably departing from and arriving at the same beach for the duration of the breeding season[12,51], which allows the island, small though it is, to be evaluated as a group of population zones. Estimates using a mark-recapture method that recorded birds returning to the island at five landing sites gave partial population estimates of approximately 700 in 1987, 425 in 1988, 620 in 1989, 450 in 1990 and 525 in 1991[87]. More recently, the most accurate island-wide population estimates have been made using a mark-recapture methodology which involved capturing penguins at four landing sites, repeating the capture process three times (2007) or four times (2008) at two to three week intervals, and evaluating the data in combination with a head-count of penguins arriving at a total of 14 sites[12]. By this method, the total population of Penguin Island was estimated to be 2369 individuals in 2007. The following year, the total population was estimated at 2069 birds. In 2011, another population estimate was obtained using a slightly modified methodology. Due to some violations in the assumptions of the models used with the 2011 data, a Robust Design model was used to compare abundance between years. With these adjustments, the comparable population estimates over a shorter time frame were 1695, 1413 and 964 penguins in 2007, 2008 and 2011 respectively (Cannell, unpubl. data). While these numbers are higher than those obtained from the years 1987 to 1991, the earlier estimates are for a part of the island only, and are therefore presumed to underestimate the total population; also, direct comparisons with the 1987 to 1991 estimates cannot be made, as the estimates were made using substantially different methodologies (see[12]). Overall, the data suggest that the population is decreasing substantially, and to make matters worse, indicators of breeding success (the number of chicks produced per pair, the

proportion of eggs that hatched, the proportion of eggs that led to fledglings, and the average mass of chicks at the time of fledging) have also declined in recent years in association with elevated sea surface temperatures, suggesting that further population decline can be expected[11].

The decline in population is attributable to a range of pressures that are reducing both the expected life span of individual birds and the breeding success of those that remain. In recent years, poorer breeding in this colony has been associated with higher sea surface temperatures and an increased Leeuwin current[11]. The Leeuwin current is a nutrient-poor seasonal current which carries water south from the tropical north, reducing the capacity of the water to support sea flora and fauna alike[11]. This effect, combined with reduced fish stocks due to commercial and recreational fishing, the disturbance of fish nurseries by water craft and their launch sites, and reduced water quality have been identified as some of the most important anthropogenic threats to the penguins' wellbeing[3]. As noted above, when local fish stocks are inadequate, penguins must forage further afield which keeps them away from their nests for longer periods and reduces overall breeding success (Cannell, unpubl. data). Other significant threats to individual penguins include trauma from watercraft strikes, entanglement in plastic detritus, and land habitat disturbances, whether intentional or inadvertent, from human visitors to Penguin Island[57].

One component of the Department's monitoring strategy is to request post mortem examinations to determine the cause of death for Little Penguins found dead in Western Australia, with a particular focus on those birds found between Perth and Dunsborough. The post mortems have been carried out by veterinary pathologists at Murdoch University Veterinary Hospital (MUVH), and, over the past ten years, the most common causes of death in this population have been found to be starvation, traumatic injuries (frequently attributed to watercraft strike) and a range of infectious conditions (Cannell *et al*, in prep). In 2011/2012, however, 12 penguins were found to have evidence of a protozoan parasite infection, principally affecting the liver and spleen, which caused significant necrosis of these tissues and which was deemed to have caused the deaths of the birds. Morphologically, the parasites in the tissues were identifiable as apicomplexans, a group of protozoa with a slightly elongate form and an eccentric nucleus. On the basis of the parasite morphology and the pattern of infection predominantly affecting the reticuloendothelial system, the infections were provisionally diagnosed as Avian malaria. Avian malaria, a haemoparasitic disease of birds caused by *Plasmodium* species parasites, has a worldwide distribution[6] and is common in captive penguin populations (see discussion below), but by virtue of presenting as an outbreak associated with an apparent spike in penguin mortality, it appeared to represent a new threat to this population. One-

off cases of suspected Avian malaria in Little Penguins had previously been diagnosed at MUVH in 2006 and 2008, though each diagnosis was made on histologic morphology alone, and neither these nor the later cases had been confirmed to be *Plasmodium* infections. A complete discussion of the cases forms the basis of Chapter 2 of this thesis.

Haemoparasitic diseases of birds

Haemoparasites are a closely related group of common vector-borne protozoan parasites infecting birds, reptiles and mammals, with the common feature that at least one stage in their lifecycles occurs in the host's erythrocytes. Avian haemoparasites include species from the families Haemospororida (e.g., *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) and Aconoidasida (e.g., *Babesia*). Infection of free-living birds with parasites from the Haemospororida has been reported worldwide and such infections appear to be common by most reports[42,43,72,80,83]. Note that the term "Avian malaria" is used to refer specifically to infections with *Plasmodium* spp. parasites, though, occasionally, infections with *Haemoproteus* spp. are also included under this general heading (for example, [73,74]). However, given that there are substantial differences in pathogenicity, vectors and host specificity, the restricted definition has been argued to be both more appropriate and more useful[83]. In this document the term will be applied to infections with *Plasmodium* spp. only; haemoproteosis and leucocytozoonosis will be used to indicate infections with *Haemoproteus* and *Leucocytozoon* respectively. Similarly, babesiosis indicates infection with *Babesia* spp.

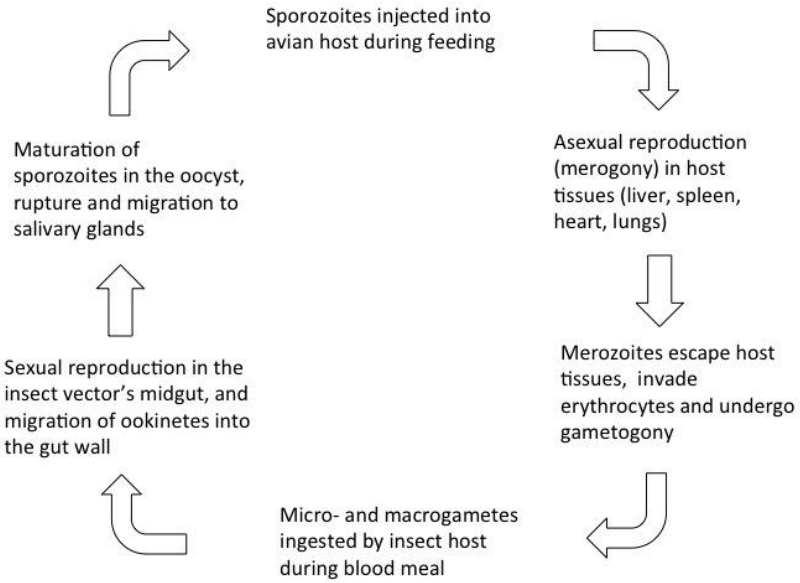


Figure 3 - Generalised life cycle of haemosporidian parasites

The haemoparasite life cycle typically involves a vertebrate and an invertebrate host. The parasites are invariably transmitted by an arthropod vector, which injects protozoal sporozoites into the vertebrate host during feeding. The sporozoites migrate through tissues and in the blood to infect macrophages and endothelial cells in various tissues (typically including liver, spleen, muscle and lung) where they reproduce as merozoites. New merozoites emerge from ruptured host cells, infect erythrocytes and develop as gametocytes, which are then ingested by the arthropod vector during a blood meal. Note that a characteristic point of distinction between *Haemoproteus* and *Plasmodium* is that *Plasmodium* parasites have additional asexual reproduction stages, which take place in erythrocytes resulting in the further production of merozoites. Sexual reproduction with the development of ookinetes and oocysts takes place in the arthropod's intestinal tract, ultimately producing schizonts which migrate to the arthropod's salivary glands ready for transmission to a new vertebrate host[6,83]. *Plasmodium* parasites are transmitted by mosquitoes, *Haemoproteus* are transmitted by ceratopogonid and hippoboscid biting flies, and *Leucocytozoon* are transmitted by Simuliid biting flies[6]. *Babesia* spp. are distinct in that they are pure red cell parasites, that is, they have no tissue phase, and they are transmitted by ticks.

The clinical signs and post mortem findings arising from *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infections are relatable to the life cycle of the parasite in the vertebrate host. Asexual reproduction of merozoites in macrophages of the liver and spleen lead to necrosis and enlargement of these tissues with an associated inflammatory response and systemic illness[5]. The erythrocyte stages induce the removal of infected erythrocytes from the circulation and haemolysis in the liver and spleen, resulting in anaemia. In some cases, post mortem examination also reveals intracoelomic haemorrhage from rupture of organs, often the liver[31]. Microscopically, the parasites are detected as small bodies encysted in the cytoplasm of macrophages or free in necrotic tissues. Parasites may also be seen in the cytoplasm of erythrocytes in blood smears or occasionally in cytological preparations (impression smears or fine needle aspirate biopsies). Speciation of the parasites may be done on erythrocyte stage (i.e., gametocyte) morphology[83] or by molecular techniques including Polymerase Chain Reaction, with or without sequencing,[85] or other specific tests such as serology. As a red cell parasite, the clinical signs of babesiosis are principally due to the destruction or removal of erythrocytes from circulation and the generation of inflammatory mediators. Typical clinical manifestations include anaemia, fever, jaundice, malaise, lethargy and anorexia[77].

Many *Plasmodium* species parasites have a low host specificity, with the result that cross-infection between avian species occurs quite commonly (though there is substantial variation in specificity between *Plasmodium* species)[6,83]. Among the most important in terms of avian disease worldwide are *Pl. relictum*, which has been reported to infect at least 419 species from 70 avian families, and *Pl. elongatum*, with a relatively restricted host range of 67 avian species[6]. *Haemoproteu*s and *Leucocytozoon* parasites, by contrast, tend to be highly host specific[6,83]. For the most part, this means that the avian hosts are relatively well adapted to these parasites and infections are usually subclinical, though not necessarily without health consequences[6,70]. Aberrant infections, however, do occur, and disease and deaths due to haemoproteosis and leucocytozoonosis have been reported in captive birds, especially in mixed aviaries and zoos where birds may be exposed to haemoparasite species which they do not normally encounter[31 20,61,63].

The prevalence of haemoparasite infections in seabirds is somewhat lower than that in other bird populations, which has been attributed to factors directly and indirectly related to reduced exposure to the invertebrate vectors, and to natural immunity[19,71,72]. Even so, penguins are among the seabirds which have been found often to have haemoparasite infections, with a mean prevalence in one review of 14.4%[72].

Haemoparasitic diseases of penguins

Avian malaria is an important disease in captive penguins. Several species of *Plasmodium* have been implicated, though the most common are *Pl. relictum*, *Pl. elongatum* or *Pl. cathemerium*[6,45]. For example, *Pl. relictum* was found to infect Magellanic Penguins (*Spheniscus magellanicus*) in the Sao Paulo zoo in Brazil[9] in an outbreak characterised by acute onset of clinical signs and a high mortality rate. Infection of a Magellanic penguin in a South Korean theme park collection has also been reported as due to a *Plasmodium* spp. infection, though the parasite was not identified to the species level[52]. *Pl. elongatum* and *Pl. relictum* were reported to cause both clinical and subclinical infections in African Black-footed Penguins kept (*Spheniscus demersus*) in the Baltimore Zoo in the USA[17], and *Pl. relictum* was associated with high mortality in Magellanic Penguins in a zoo in Des Moines, USA[32].

There have also been a number of reports of infections with haemosporidian parasites in free-living penguins, though their association with disease is less clear than it is in captive penguins, which are inevitably under closer scrutiny. Deaths attributed to *Pl. juxtanucleare* have been reported in five African Black-footed penguins that had been admitted to a rehabilitation facility in South Africa[35].

Studies reporting the seroprevalence of plasmodium infections have been published in Jackass Penguins (aka African Black-footed Penguins, *Spheniscus demersus*) from South Africa[33], and in Galapagos Penguins (*Spheniscus mendiculus*) from the Galapagos Islands[64]. The average seroprevalence in the Jackass Penguins was 39%, with a range in several studies of samples taken from wild and rescued birds of 20% to 62%. In the Galapagos Penguins, 97.2% (176/181) of the penguins tested positively using an ELISA assay to detect plasmodium antibodies. PCR screening of blood samples from the same population, however, returned only 9.4% (17/181) positive results. In another study of wild Galapagos penguins, *Plasmodium* parasites were detected by PCR of blood samples, reporting a prevalence of 5% (19/362)[54]; in this survey, the DNA from one of the penguins which returned a positive PCR was sequenced to identify a *Haemoproteus* infection rather than *Plasmodium*. In 1999, Jones and Shellam published a survey of haemoparasites in wild living penguins from locations in Antarctica, South America and Australia, including the Little Penguins of Penguin Island[46]. They examined blood smears from 194 penguins of 4 species (Emperor Penguin (*Aptenodytes forsteri*), Little Penguin, Humboldt Penguin (*Spheniscus humboldti*) and Adelie Penguin (*Pygoscelis adeliae*)) and did not detect haemoparasites in any of the smears. Evidence as to the presence of *Plasmodium* infection in Yellow-Eyed Penguins (*Megadyptes antipodes*) in New Zealand identifies seropositivity for *Plasmodium* antibodies in some studies, and negative PCR findings in others[80]. Overall, these results suggest that, in many penguin populations, exposure to *Plasmodium* is in fact high, but that parasitaemia, and perhaps also clinical disease, is far less common.

Leucocytozoon has been reported in Yellow-Eyed Penguins from New Zealand, with the prevalence of infection, as measured by PCR, varying by location from 74% to 11%[4,37]. In these birds, the infection is considered to be of generally low pathogenicity; occasional individual deaths are attributed to leucocytozoonosis, as are some mass mortality events, though the causal connection linking the parasites to the deaths is far from certain. *Leucocytozoon* has also been reported in wild Fiordland Crested Penguins (*Eudyptes pachyrhynchus*) in New Zealand[29]. In captive penguins, infection with *Leucocytozoon* has been reported only in 3 Macaroni penguins (*Eudpytes chrysolophus*) from an English zoo, and then the infection was not associated with disease, but was detected during an investigation into the death of one other bird from this collection due to a *Plasmodium* infection[69]. *Haemoproteus* infection has not been reported in penguins with two exceptions: the single Galapagos penguin noted above, and recently in four Little Penguins from Penguin Island[14], of which there will be more discussion in chapter five of this thesis.

Other protozoan parasites infecting penguins

The haemosporidian parasites discussed to this point belong to the phylum Apicomplexa, a taxon in which the members have in common an apical complex consisting of rhoptries, micronemes, a conoid and a polar ring associated with microtubules[15]. Among apicomplexan parasites other than the Haemospororida (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) few have been reported to cause infections in penguins. Probably the most notable is a piroplasmid infection in African Black-Footed Penguins, in which *Babesia peircei* is considered to be an enzootic infection[27]. There is also a single report of Little Penguins in Australia infected with a parasite which was morphologically identified as consistent with *Babesia* (cited in Duignan, 2001[25]), though further details are lacking. Infections with *Theileria* have not been reported in penguins.

One further apicomplexan parasite which has been reported to cause disease in a Little Penguin is *Toxoplasma gondii*[56]; notably, the infection in this case resulted in marked hepatitis and splenitis, and was acutely fatal. While it is not a haemoparasite as such, this protozoan can infect the nucleated erythrocytes of avian species[78], a feature which does not occur in mammalian species. While reports of toxoplasmosis in penguins are rare, it has been reported to cause fatal infections in juvenile Black Footed penguins[71]. The exposure of seabirds to *Toxoplasma* would be expected to be low, as the definitive host of this parasite is the cat. Curiously, however, a survey of Galapagos Penguins found that penguins living on an island inhabited by cats (Isabela Island) had a lower seroprevalence for *Toxoplasma* than those living on an adjacent, cat-free island (Fernandina Island)[19].

Summary

The evidence from the literature suggests that haemoparasite infections in wild penguins are not rare, but that the pathogenicity of the infections is probably low in adapted populations. Exposure of naïve populations to new infections, however, as has been observed in many captive penguin populations, may result in overt disease and deaths. With regard to the Little Penguins of Penguin Island, the temporal cluster of fatal protozoan infections in this wild population is suggestive of the introduction of the infection to non-adapted hosts. The true incidence of disease in the population is unknown, as the sample of birds found and presented for post mortem in a timely manner is likely to represent a very small proportion of overall deaths in this group. In general terms, a new infection might be introduced to a population in several ways. These include: by the introduction of a new host carrying its own burden of infectious organisms that can be spread to other species; by a

migratory host already present in that environment which has itself become exposed to a new infection in another location from which it migrates; or by altered climatic conditions that permit the extension of a new vector into the environment[28].

The aims of this project are three-fold:

1. to document the post mortem findings that are associated with this protozoan infection
2. to identify the parasite at least to the level of genus, and
3. to commence collection of data intended to assess the prevalence of the disease in the live penguin population and in potential avian hosts other than the penguins.

2. Post mortem examinations

Introduction

On behalf of WA government bodies and academic researchers, routine post mortem examinations of Little Penguins have been conducted by Murdoch University Veterinary Hospital's (MUVH) pathology department since at least 2001. In many instances, the birds have been found dead on or around Penguin Island, though they are also found on Perth beaches and in other locations as far away as Busselton, approximately 190km to the south. Sometimes, penguins have been found unwell or moribund, and have been taken to private veterinary clinics for assessment followed by treatment or euthanasia according to the attending veterinarian's judgement. For the most part, however, the time period elapsed between the bird's death and its discovery and submission for post mortem is unknown, and consequently many of the birds are severely autolysed on presentation, greatly reducing the ability to diagnose a cause of death or to reliably detect gross and histologic lesions. In addition, many penguins had been stored frozen prior to presentation for post mortem, in which case artefacts associated with freezing and thawing also distort the tissues. Despite these limitations, the examination often permits useful conclusions to be drawn, or at least rules in or out common causes of death such as starvation and major traumatic injury.

During the latter months of 2011 (November and December), four penguins were found within the space of 2 months to have died with similar, distinctive lesions. In each case, the birds were diagnosed to have died from, or at least to have tissue changes consistent with, Avian malaria (though it was noted in each case that this diagnosis was provisional rather than confirmed). A review of penguin post mortems since 2001 revealed that a similar infection had been presumptively diagnosed in a single case in 2006, and another in 2008. Subsequently a further 8 cases were diagnosed, out of a total of 40 penguins submitted, in 2012, and 2 more cases were diagnosed from 23 post mortems in 2013. An abridged version of each post mortem report from these cases is presented here to encompass the relevant information, and the major findings are summarised in Table 1.

Materials and methods

Routine penguin necropsy procedure

From 2011 onwards, post mortems were conducted in accordance with a standardised protocol devised jointly between Dr Belinda Cannell and the veterinary pathologists at MUVH (see appendix 1). Prior to 2011 (that is, in the 2 cases from 2006 and 2008) the post mortem was conducted according to the experience and judgment of the pathologist on duty at the time of submission.

Under the standardised protocol, each penguin was weighed and examined externally for evidence of injuries and ectoparasites. A body condition score was estimated based on the bird's bodyweight, degree of pectoral musculature and the presence of subcutaneous and abdominal fat stores, with a score of 1 indicating emaciation and a score of 5 indicating obesity. In most cases, swabs were made of the oropharynx and cloaca, placed in viral transport medium; these were submitted to the Department of Agriculture and Fisheries Animal Health Laboratory in Kensington, Western Australia for PCR assay for Avian Influenza virus and Newcastle Disease virus antigen. Bacteriology was not included in the protocol as the great majority of submissions were found well after the time of death and post mortem bacterial growth would have made the results virtually uninterpretable. Penguins were placed in dorsal recumbency and a midline incision was made from the beak to the cloaca, reflecting the skin to the lateral midline. The coelomic cavity was incised and the gross position of the internal organs was assessed. Where present, the abdominal fat pad surrounding the cloaca in the coelomic cavity was dissected free and weighed, then wrapped in aluminium foil and stored at -20°C. The sternum was removed by cutting through the ribs dorsally and through the clavicles and humeri bilaterally. The heart was removed and assessed grossly by transecting the great vessels near their insertion. One half of the heart was fixed in 10% formalin, and the other half was wrapped in aluminium foil and stored at -20°C. The liver was removed and weighed, then thinly sliced to identify possible lesions in the parenchyma. At least one section of liver was fixed in formalin, and a second sample was stored in aluminium foil at -20°C. The tongue was externalised ventrally by incising through its attachments on the medial aspects of the mandibles, then the tongue, oesophagus and trachea together were retracted after cutting through the hyoid apparatus. The oesophagus was dissected free from the trachea, and the entire gastrointestinal tract was removed. The oesophagus and stomach were opened and inspected to assess for the presence of gastric parasites (in particular the gastric nematode *Contracaecum*). The spleen was inspected, weighed and one half was fixed in formalin while the other half was stored at -20°C. The kidneys were removed and inspected, one entire kidney fixed in formalin, the other stored at -20°C. The

gonads were inspected. The brain was removed and half was fixed in formal, the other half stored at -20°C.
In two cases (11/627 and 11/628), samples of liver and spleen were placed in Optimal Cutting Temperature compound, snap frozen in liquid nitrogen, and then stored at -80°C. For three cases (12/362, 12/375, 12/385), samples of liver and spleen were fixed in glutaraldehyde and stored at 4°C in preparation for electron microscopy.

Unless the tissues were judged to be too autolysed, routine histology (4µm tissue sections stained with haematoxylin and eosin) was performed on liver, spleen, heart, lung, kidney and brain. Histology of other tissues, including skeletal muscle, skin and proventriculus was included if gross lesions had been detected.

The weights of the liver, spleen and abdominal fat pad were interpreted with reference to a published survey of *E. minor* organ weights compiled from birds submitted for post mortem from the Otago coast in New Zealand's South Island[39]. In this paper, the weights of these organs, along with heart, kidney and genitalia, are tabulated as normal organ weights for adult and juvenile Little Penguins. The reported weights for spleens in the adult penguins range from 0.23 to 5.94g, a 25.8-fold range, which the author justifiably declares to be biologically uninterpretable. In all probability, the larger spleens were not normal at all. Organs from animals which have died naturally cannot be assumed to be normal, and splenomegaly is associated with many disease processes. With this ambiguity in mind, some spleens in the cases compiled below were not identified as being of abnormal size because they fell within this reported range, while others of similar size were reported as being enlarged, essentially as a subjective judgement made by the pathologist, particularly if other gross pathological changes were noted. For this reason, 'splenomegaly' is a consistent finding inconsistently reported in these reports. To a lesser degree, but for comparable reasons, hepatomegaly is an inconsistent finding inconsistently reported.

Results

All penguin post mortems conducted at Murdoch University Veterinary Hospital from 2001 to 2013, the period for which a searchable database exists, were reviewed, a total of 168 cases. The following are those which were diagnosed with protozoal infections of spleen and/or liver. In the second case, 08/1075, the pathologist's comment included an observation that the protozoa are most likely *Plasmodium*, the causative agents of Avian malaria, and in subsequent cases this attribution was usually followed. The cases are presented in order of increasing pathology accession number, that is, the order in which they were accepted at MUVH. Many of the birds had been

frozen for some time prior to examination, so the sequence does not necessarily indicate the order of deaths.

Case1: 06/1172

Gross findings:
The bird was in good body condition and there were mild autolytic changes. There was mild splenomegaly.

Histopathology:
Spleen: there is moderate multifocal necrosis of the splenic parenchyma and moderate multifocal to coalescing histiocytosis. Within the foci of necrosis are aggregates of necrotic cells with pyknotic or karyorrhectic nucleus, shrunken and fragmented densely eosinophilic cytoplasm, eosinophilic proteinaceous material and abundant oval to round protozoal organisms, 1-2µm across with an apical densely basophilic nucleus. The protozoa are also seen within the cytoplasm of macrophages.
Liver: there is mild multifocal random necrosis of hepatocytes surrounded by small aggregates of Kupffer cells and rare heterophils. The necrotic hepatocytes have pyknotic or karyorrhectic nucleus and shrunken densely eosinophilic cytoplasm. There are moderate numbers of protozoa within the necrotic foci. They are also seen within the cytoplasm of normal hepatocytes and Kupffer cells, particularly around periportal areas.
Protozoa were also observed surrounding a pericardial artery, in the pulmonary interstitium and in the adipose tissue surrounding the carotid artery in the thoracic inlet.

Final comment:
The pathologist's final comment indicated that protozoan infections of Little Penguins had not previously been reported in Australia, and that infection with *Plasmodium juxtanucleare* had been reported in *Spheniscus demersus* (the African Black Footed Penguin) (J Zoo Wildl Med. 2003 Sep;34(3):250-5). The possibility of molecular assays was raised, but there is no indication that this was followed through. The term 'Avian malaria' was not used.

Case 2: 08/1075

Gross findings:
External examination: Blood was seen on the surface of the feathers around the mouth and on the chest. The eyes were absent. Body weight was 1.3 kg. The bird was well feathered and the skin was very thick with a layer of subcutaneous fat. There were no penetrating wounds into the skin.
Alimentary system: Overall the liver felt soft and was fragile, weight was 69.42g. The right liver lobe had focal metallic yellow speckling in a linear arrangement extending from the lobe edge to just over half of the lobe length (*urate precipitate on the capsular surface*). The underside to the

same liver lobe was diffusely discoloured greenish black colour (*post-mortem pseudomelanosis*). All other lobes were also discoloured but only along the edges. The bile duct was elongated (8.5 cm) and distended with bile.

Histopathology:
Spleen: There are multifocal aggregates of macrophages with one to four intracytoplasmic bodies resembling apicomplexan parasites that are approximately 2µm diameter with a dense eccentrically located central body.
Heart: There are a few foci where cardiac myocytes are disrupted by aggregates of intracytoplasmic apicomplexans.
Liver: There is multifocal degeneration and necrosis of hepatocytes with intracytoplasmic apicomplexan parasites, occasionally arranged in a ring form. There is moderate diffuse extramedullary haematopoiesis.
Lung: There are diffusely increased numbers of macrophages in the interstitium, some with intracytoplasmic apicomplexans.

Final Comment:
The intracytoplasmic bodies resembling apicomplexan parasites are most likely the tissue phase of *Plasmodium* sp. (the causative agents of avian malaria). However, [it is surprising] to find disease due to *Plasmodium* species, because a penguin that is a member of a resident population would not normally develop severe clinical disease and succumb to the parasite species present in its usual range. Clinical avian malaria is more usually encountered in zoological collections where penguins are kept in a foreign environment. Alternative aetiologic agents include other apicomplexan parasites such as *Toxoplasma* or *Sarcocystis* sp. In addition the appearance of the intra-cytoplasmic bodies in semi-putrefied tissue sections is indistinguishable from amastigotes of *Leishmania* or *Trypanosoma* sp. It will be interesting to monitor the population for any clinical disease or deaths with similar findings.

Case 3: 11/484

Gross findings:
The bird weighed 1350g and there was a large amount of subcutaneous adipose tissue present. The alimentary system contained no ingesta or digesta.

Histopathology:
The kidney and liver sections had moderate to marked autolytic changes. Many hepatocytes contained 1-2 medium, occasionally large, clear vacuoles in the cytoplasm (vesicular change, hepatic lipidosis).

Final comment:

The absence of ingesta/digesta in the gastrointestinal tract and hepatic lipidosis are supportive of acute starvation as the cause of death. No further evidence of underlying disease was found.

Note: during a later review of post mortem findings conducted by Dr Belinda Cannell, it was considered that the high bodyweight and abundant adipose tissue in this bird made the diagnosis of death due to starvation unlikely, the more so given that Little Penguins regularly endure periods of inanition during breeding and moulting without apparent adverse physiological consequences other than weight loss. On reviewing the slides, it was observed that there were multifocal histiocytic infiltrates in the liver, and that in these areas there were numerous 1-2µm ovoid organisms, free and within macrophages. There was no spleen sample to evaluate, nor was there any remark made on the presence of splenomegaly or measurement made of the weight of the spleen. Though the evidence is incomplete, the death of this penguin is now presumed to have been due to the protozoal hepatitis, rather than starvation.

Case 4: 11/624

Gross findings:
The penguin was in reasonable to good body condition with subjectively adequate to good muscle bulk and a convex pectoral profile; it weighed 1370g. The spleen was markedly enlarged (spleen weight = 9.61g; congestion) and had a granular appearance to the cut surface, with multitudinous pinpoint off-white foci scattered throughout the parenchyma (lymphoid hyperplasia e.g. secondary to chronic antigenic stimulation versus neoplasia, necrosis or inflammatory cellular infiltrate).

Histopathology:
Lung (left and right): The pulmonary vasculature is diffusely hyperaemic. Autolysis and freezing has somewhat disrupted the appearance of the parenchyma; however it appears that the pulmonary interstitium is diffusely hypercellular owing to the presence of increased numbers of inflammatory cells, which appear to be a mixture of predominantly macrophages with lesser numbers of granulocytes (heterophils). Occasionally the cytoplasm of macrophages contains several intracytoplasmic bodies resembling apicomplexan parasites (notably the tissue phase of *Plasmodium* spp.).
Spleen: Autolysis and freezing has somewhat disrupted the appearance of the parenchyma. There is widespread, multifocal to coalescing splenic parenchymal necrosis with an associated marked multifocal to coalescing increase in heterophils and macrophages. Numerous macrophages and many heterophils contain numerous intracytoplasmic bodies resembling apicomplexan parasites (notably the tissue phase of *Plasmodium* spp.; presumably having phagocytosed merozoites that have been released from schizonts).
Liver: There is diffuse hyperaemia. Autolysis and freezing has somewhat disrupted the appearance of the parenchyma. Numerous individual macrophages (Kupffer cell hypertrophy) are scattered throughout the sinusoids and parenchyma and they frequently contain intracytoplasmic bodies

resembling apicomplexan parasites (notably the tissue phase of *Plasmodium* spp.). Frequently the hepatocytes exhibit mild microvesicular vacuolar hepatopathy and both they and Kupffer cells contain a small amount of golden-brown pigment (haemozoin/malarial pigment, haemosiderin).

Final Comment:
Histopathology of the liver, lungs and spleen in this case revealed the presence of macrophages containing intracytoplasmic bodies resembling apicomplexan parasites; they resemble the tissue form of *Plasmodium* spp. These protozoal organisms are associated in this case with severe splenic necrosis and inflammation, and the findings are consistent with disseminated Avian Malaria. This case bears close resemblance to pathology no. 08/1075. It would be interesting to know if there has been a recent sudden increase in mortality, particularly in birds in relatively good body condition that could be attributable to a possible outbreak of *Plasmodium* spp.

Case 5: 11/626

Gross findings:
Bodyweight 1105.3g. The spleen was subjectively moderately enlarged (7.53g) and was friable and bloody. The liver appeared mildly enlarged and weighed 61.19g; there were multifocal pinpoint white spots on all lobar capsular surfaces (necrosis, fibrosis, foci of cellular infiltrate i.e. hepatitis).

Histopathology:
Liver: There are numerous multifocal, randomly placed foci of hepatocellular necrosis. Degenerate leukocytes within these areas as well as the hepatocytes themselves appear to contain intracytoplasmic bodies resembling apicomplexan parasites. There is a multifocal to coalescing, mild to moderate, mixed periportal inflammatory infiltrate. Some of the leukocytes appear to contain intracytoplasmic bodies resembling apicomplexan parasites.
Spleen: There is widespread, multifocal to coalescing splenic parenchymal necrosis with an associated marked multifocal to coalescing increase in degenerate leukocytes (probably heterophils and macrophages). These degenerate leukocytes contain numerous intracytoplasmic bodies resembling apicomplexan parasites.

Final Comment:
Histopathology of the liver and spleen in this case revealed the presence of degenerate leukocytes (likely macrophages) containing intracytoplasmic bodies resembling apicomplexan parasites; they resemble the tissue form of *Plasmodium* spp. Numerous hepatocytes also contain the same structures. These protozoal organisms are associated in this case with hepatic and splenic necrosis and inflammation, and the findings are consistent with disseminated Avian Malaria. This case bears close resemblance to pathology no. 08/1075 and 11/624 (the latter penguin submitted and post-mortemed in this batch of penguins).

Cases 6 and 7: 11/627 and 11/628

Gross findings:
Body weights = 1160g (11/627) and 1225g (11/628). The liver in the case of both penguins appeared mildly enlarged and weighed 79.73g (11/627) and 63.73g (11/628) respectively; there were a few pinpoint white spots on each lobar capsular surface in the case of both livers (not evident within the parenchyma) (necrosis, fibrosis, foci of cellular infiltrate i.e. hepatitis). The spleens from both penguins appeared normal in size (5.03g, 11/627 and 4.21g, 11/628) and were friable (PM change, necrosis).

Histopathology:
Liver: The livers from both birds exhibit numerous small multifocal randomly scattered foci of hepatocellular necrosis with an associated mild, multifocal to coalescing patchy mixed degenerate leukocyte infiltration. Scattered aggregates of hepatocytes as well as associated degenerate leukocytes contain intracytoplasmic bodies resembling apicomplexan parasites.
Spleen: The spleens from both birds exhibit widespread, multifocal to coalescing splenic parenchymal necrosis with an associated marked multifocal to coalescing increase in degenerate leukocytes (probably heterophils and macrophages). These degenerate leukocytes contain numerous intracytoplasmic bodies resembling apicomplexan parasites.
Heart: 11/627 was unremarkable. 11/628 exhibited a focally extensive area of acute myocardial necrosis with an accompanying mild mixed inflammatory infiltrate. Numerous cardiac myocytes in this area as well as the associated leukocytes contain intracytoplasmic bodies resembling apicomplexan parasites.

Final comment:
Histopathology of the liver and spleen in both of these cases (and, additionally, the heart in 11/628) revealed the presence of degenerate leukocytes (likely macrophages) containing intracytoplasmic bodies resembling apicomplexan parasites; they resemble the tissue form of *Plasmodium* spp. Numerous hepatocytes in both cases, as well as cardiac myocytes in the case of 11/628 also contain identical organisms. These protozoal organisms are associated in these 2 cases with hepatic and splenic necrosis and inflammation (and, in the case of 11/628, a focus of cardiac muscle necrosis), and the findings are consistent with disseminated Avian Malaria. This case bears close resemblance to pathology no. 08/1075, 11/624 and 11/626 (the latter 2 penguins submitted and post-mortemed in this batch of penguins).

Case 8:12/020

Gross findings:

Bodyweight 1230g. The pectoral muscles formed a convex surface and all bony prominences were adequately covered by soft tissues and muscle, giving the bird a body condition score of 3/5.
Spleen - the spleen measured 3.1 x 2 x 1.3cm and weighed 3.8grams.

Histopathology:
Liver – within the parenchyma there are multifocal, small to moderate (up to 1mm diameter) approximately circular areas in which hepatocellular detail is lost, leaving mostly amorphous eosinophilic debris and pyknotic and karyorrhectic nuclei (necrosis). Within the necrotic areas there are many small extracellular organisms resembling apicomplexan parasites. The organisms measure approximately 1-2µm in diameter with a dense central nucleus surrounded by a round, clear capsule, and they are often present in small clusters or chains (presumptive *Plasmodium* spp.). Surrounding the smaller necrotic foci, but mostly absent from the larger ones, are infiltrations of lymphocytes, plasma cells and macrophages. In some areas, organisms can be seen in macrophages. Throughout the section and mostly within hepatocytes or sinusoids, there is a moderate amount of slightly refractile, yellow-brown pigment.
Spleen – throughout the section there is a diffuse loss of all red pulp architecture, with lymphocytes, plasma cells and macrophages infiltrating in greater or lesser numbers against a background of amorphous eosinophilic debris and pyknotic and karyorrhectic nuclei (necrosis). The section contains a multitude of extracellular organisms as described above. The white pulp structure is lost.

Final Comment:
The severe tissue necrosis seen in the spleen and liver are presumed to have resulted in a state of acute shock, causing the death of this otherwise apparently healthy bird. A presumptive diagnosis of Avian Malaria is made based on the morphological characteristics of the observed organisms in the liver and spleen, though molecular tests are required for definitive diagnosis. This case appears similar to that of a previous case, pathology number 11/624 reported on 16/11/2011, in which it was noted that Avian malaria (*P. relictum*) is a common cause of captive penguin mortality in the Northern hemisphere and in African Black-Footed Penguins; clinical disease due to *Plasmodium* spp. has been reported in free-living Blue (aka Little) Penguins previously[25]. Also, Avian Malaria has been reported to cause mortalities with a rapid clinical course in Magellanic Penguins in captivity[9].

Case 9: 12/021

Gross findings:
Bodyweight 1300g, body condition score of 3/5. The liver weighed 58.6g and the spleen weighed 3.3g.

Histopathology:

Liver – the architecture and cytological detail of the liver are diffusely lost (autolysis). Scattered throughout the parenchyma, there are occasional small extracellular organisms resembling apicomplexan parasites. The organisms measure approximately 1-2µm in diameter with a dense central nucleus surrounded by a round, clear capsule, and they are at times present in small clusters or chains (presumptive *Plasmodium* spp.).
Spleen – the architecture and cytological detail of the spleen are diffusely lost (autolysis). There are occasional small clusters of organisms as described above.

Final Comment:
Marked autolytic changes prevent any reliable histological assessment of the tissues examined. The significance of the splenic and liver parasites is difficult to judge. Recent similar cases have seen such parasites, likely the agents of Avian Malaria, in areas of splenic and hepatic necrosis. However, in this case distinguishing possible pre mortem necrosis from post mortem autolysis is impossible. In addition, the numbers of parasites observed in this case are relatively low. The infection may have been subclinical, or it may have been a contributing factor in the bird's death. On the available evidence, the cause of death cannot be ascertained, though starvation, trauma and/or predation can be ruled out.

Case 10: 12/338

Gross findings:
With a bodyweight of 1.19kg and good soft tissue coverage of the keel bone, a body condition score of 3/5 was given. The liver weighed 62.4g. The spleen was uniformly enlarged, weighing 6.9g

Ancillary Tests: Newcastle disease virus antigen suspect positive.

Histopathology:
Liver and spleen: while both samples are moderately to severely autolysed, areas of loss of all cellular detail can be identified amongst the autolysed tissue (antemortem necrosis), these accompanied by many small (1-2µm), round bodies consisting of a densely basophilic centre surrounded by a non-staining capsule (apicomplexan parasites).

Final Comment:
The protozoal organisms observed in the liver and spleen are similar to those reported for case 12/020 among others. The organisms are considered to be most consistent with *Plasmodium* species, though this diagnosis is yet to be confirmed. In any case, acute necrotising splenitis and hepatitis with probable systemic consequences is considered to be responsible for the bird's death.

Case 11: 12/362

Gross findings:
Bodyweight 1240g. Good soft tissue coverage of bony prominences, convex pectoral muscles, giving a body condition score of 3.5/5. At 59g, the liver comprised 4.8% of bodyweight, putting it at the upper limit of the reported range for adult males (range 2.8 to 4.8%[39]). On the capsular surface and the cut surfaces throughout the parenchyma, there were multiple, randomly distributed, 0.5 to 1mm, tan to white, moderately well-delineated flat discolourations (necrosis). At 5.9g, the spleen was at the upper end of the reported range for spleen weights in the little penguin. Measuring 33 x 24 x 12mm, it appeared uniformly enlarged. On the capsular surface there were similar lesions to those seen in the liver.

Histopathology:
Liver – throughout the parenchyma, there are frequent foci, occasionally extensive, in which the hepatic architecture has been lost and replaced by amorphous eosinophilic debris containing pyknotic and karyorrhectic nuclei (necrosis). Within these foci there are abundant small (2-4µm), round to ovoid organisms, each with an eccentric nucleus (protozoal parasites). Primarily around blood vessels, but also scattered throughout the parenchyma, there are moderate to marked infiltrates of lymphocytes and macrophages with lesser numbers of heterophils. Away from the necrotic and inflammatory foci, the hepatocytes are moderately swollen with poorly defined cytoplasmic margins and compression of sinusoids.
Spleen – the splenic parenchyma is a virtually uninterrupted network of small to large necrotic foci containing myriad protozoal parasites as described above. The remainder of the tissue consists of lymphocytes, macrophages, erythrocytes and islands of intact splenic cords.
Heart – there are sporadic small foci in which the cardiac myocytes are hypereosinophilic with pyknotic nuclei, and which are accompanied by small clusters of protozoal parasites as described above.

Final Comment:
The severe necrotising splenitis and hepatitis with a superabundance of protozoal parasites will undoubtedly have resulted in a systemic inflammatory response leading to a state of shock and ultimately to the death of the penguin. Morphologically, the parasites are consistent with *Plasmodium* spp. as has been previously reported, though this diagnosis remains to be confirmed.

Case 12: 12/375

Gross findings:
The penguin was in reasonable to good body condition with subjectively adequate to good muscle bulk and a convex pectoral profile; it weighed 1300g. The liver was markedly diffusely enlarged (liver weight = 73g) with diffusely rounded edges. Disseminated over the capsular and cut surfaces

were innumerable pinpoint to 3mm cream to white flat spots (necrosis, inflammation)(Fig 1.) The entire liver was extremely friable, rupturing easily in the process of gentle removal. The spleen was moderately enlarged, extremely friable (spleen weight = 6.62g) and had a granular appearance to the cut surface, with myriad pinpoint to 3mm cream to white foci scattered throughout the parenchyma and over the capsular surface (necrosis, inflammation). On the heart, there were multifocal (~6-7) pinpoint to 2mm pale pink to cream flat discolourations over the surface of both ventricles, as well as a focally extensive area of coalescing 3mm long linear streaks; these did not grossly appear to penetrate into the myocardium for any appreciable distance (necrosis, inflammation). The parietal pericardium is diffusely slightly opaque (inflammation). The air sacs appear diffusely opaque white and slightly thickened (inflammation).

Figure 1 – case 12, enlarged liver with multiple, pinpoint cream to white spots

Histopathology:

Spleen: There is widespread, multifocal to coalescing, severe splenic parenchymal necrosis (~40% of the parenchyma is necrotic) with an associated marked multifocal to coalescing increase in heterophils and macrophages (which appear degenerate in the worst areas) as well as multifocal to coalescing haemorrhage. Numerous macrophages and many heterophils contain numerous intracytoplasmic bodies resembling apicomplexan parasites (notably the tissue phase of *Plasmodium* spp.; presumably having phagocytosed merozoites that have been released from schizonts); myriads of the same organisms are also free in the tissue, often at the periphery of the necrotic areas and appear well-preserved.

Liver: The tissue appears well-preserved. There are numerous multifocal, randomly placed foci of hepatocellular necrosis (occupying ~20% of the parenchyma). Degenerate leukocytes within these

areas as well as the hepatocytes themselves appear to contain intracytoplasmic bodies resembling apicomplexan parasites; the parasites are often free within and on the periphery of the necrotic tissue also. There is a multifocal to coalescing, moderate, mixed periportal inflammatory infiltrate as well as random intrasinusoidal extending to intraparenchymal mixed infiltrate; the cell population appears largely composed of heterophils and macrophages. Some of the leukocytes appear to contain intracytoplasmic bodies resembling apicomplexan parasites.
Heart: There are numerous multifocal small foci of heterophilic and histiocytic inflammation separating individual/clusters of cardiac myocytes; many of the macrophages contain apicomplexan parasites as seen in the liver/spleen and some of these appear free as well. Occasional individual/small aggregates of cardiac myocytes are necrotic, fragmented and are in the process of being phagocytosed by macrophages; heterophils are also present, and again, apicomplexan parasites are seen within the cytoplasm of macrophages in these areas.
Air sacs and parietal pericardium: Within the fibrous connective basement tissue of the air sac there is a small focal area of histiocytic and heterophilic inflammatory infiltrate. A couple of histiocytes appear to contain a few intracytoplasmic objects resembling the apicomplexan parasites seen in the liver, spleen and myocardium; however there is no notable necrosis as seen elsewhere.
Lungs: There is diffuse hyperaemia and diffuse increased cellularity due to an inflammatory infiltrate which is predominantly heterophilic and histiocytic. Rare individual macrophages scattered amongst the parenchyma contain 1-2 apicomplexan organisms. There are occasional small areas of coagulative necrosis focussed on the interstitium; and in these areas and/or at their periphery, numerous free as well as intracytoplasmic apicomplexans can be seen.
Pectoral skeletal muscle (D): There are rare individual degenerate myofibres accompanied by a mild histiocytic infiltrate localised solely to these areas; apicomplexans are not seen associated with these areas.

Final comment:
Histopathology of the liver, spleen, heart, lungs and possibly also air sac (organism numbers were low and the lesion was small so difficult to examine adequately) in this case revealed the presence of intracytoplasmic bodies (particularly within histiocytes but also seen in hepatocytes; not, however, in erythrocytes) resembling apicomplexan parasites; they resemble the tissue form of *Plasmodium* spp. These protozoal organisms are associated in this case with hepatic, splenic, cardiac and pulmonary necrosis and inflammation, and the findings are consistent with disseminated Avian Malaria. This case bears close resemblance to several other cases seen through our diagnostic service from this penguin colony recently. Thankfully the tissues from this case were well-preserved and the carcase had not been frozen; various samples for future investigative techniques are available.
Avian malaria (*P. relictum*) is a common cause of captive penguin mortality in the Northern hemisphere and African Black-Footed Penguins; clinical disease due to *Plasmodium* spp. has been reported in free-living Blue (aka Little) Penguins previously; although typically, clinical avian malaria is most often seen in captivity. As the amastigotes of *Leishmania* and *Trypanosoma* spp can

appear morphologically very similar to *Plasmodium* spp, molecular testing is required for definitive speciation of the parasite.

Case 13: 12/385

Gross findings:
With a body weight of 1175g, convex pectoral musculature and good soft tissue coverage of all bony prominences, the bird had a body condition score of 3/5. The liver was moderately large (60.73g) and on the capsular surface there were multiple 0.5–1mm flat, cream lesions (necrosis). The spleen was moderately to markedly enlarged (3.78g) with a mottled red to dark red capsular surface. On the epicardial surface of the heart, there were several, linear, 5-10mm pale red discolourations (necrosis, epicarditis).

Histopathology:
Liver – scattered randomly throughout the parenchyma, there are areas in which the hepatocytes are swollen and eosinophilic with condensed and fragmented nuclei (necrosis). Associated with the regions of necrosis are small numbers and occasional aggregates of small (2-4µm), round to ovoid organisms, each with an eccentric nucleus (protozoal parasites).
Spleen – the splenic parenchyma comprises large numbers of histiocytic cells. In many small patches, affecting approximately 30-40% of the tissue, the cells are swollen and eosinophilic with condensed and fragmented nuclei (necrosis). Protozoal organisms as described above are present in small to moderate numbers throughout the tissue.
Heart – in occasional small patches, cardiac myocytes are swollen and eosinophilic with fragmented nuclei; these patches invariably contain small numbers of protozoal parasites as described.

Final comment:
The penguin's death is attributable to a state of shock arising from the extensive splenic and hepatic necrosis associated with an acute protozoal infection. Morphologically, the parasites are consistent with *Plasmodium* sp. as has been previously reported, though this diagnosis remains to be confirmed.

Case 14: 12/473

Gross findings:
With a body weight of 1030g, convex pectoral musculature and good soft tissue coverage of all bony prominences, the bird had a body condition score of 3/5. The liver was moderately enlarged (56.4g), and randomly scattered over the capsular surface and extending into the parenchyma there were multiple 0.5–2mm flat, cream areas of discolouration (necrosis). The spleen was moderately to markedly enlarged (4.6g) and was dark red with speckled tan areas of discolouration on the capsular surface (necrosis) (Fig 2.)

Figure 2 – case 14, enlarged spleen with speckled tan areas of discolouration

Histopathology:
Liver – scattered randomly throughout the parenchyma, there are multiple, randomly-distributed patches in which the hepatic architecture is lost and the tissue replaced by amorphous eosinophilic material containing condensed and fragmented nuclei (necrosis). Associated with the regions of necrosis are large numbers of small (2-4µm), round to ovoid organisms, each with an eccentric nucleus (protozoal parasites), which are either free or, occasionally, within the cytoplasm of an hepatocyte.
Spleen – in multiple, coalescing patches of varying size, and affecting approximately 60% of the splenic parenchyma, the normal architecture is lost and replaced by eosinophilic material containing condensed and fragmented nuclei (necrosis). Myriad protozoal organisms as described above are present throughout the necrotic tissue.
Cardiovascular system:
Heart – infrequently through the myocardium, there are small clusters of protozoal parasites as described, with no clear evidence of a tissue response.

Final comment:
The penguin's death is attributable to a state of shock arising from the extensive splenic and hepatic necrosis associated with an acute protozoal infection. Morphologically, the parasites are consistent with *Plasmodium* sp. as has been previously reported, though this diagnosis remains to be confirmed.

Case 15: 12/517

Gross findings:

With a body weight of 1200g, convex pectoral musculature and good soft tissue coverage of all bony prominences, the bird had a body condition score of 3/5. At 5.62% of bodyweight (reference range 2.30 – 5.34), the liver was moderately enlarged; randomly scattered over the capsular surface and extending into the parenchyma there were multiple 0.5–2mm flat, cream areas of discolouration (necrosis). The spleen was markedly enlarged (6.18g) and was dark red with speckled tan areas of discolouration on the capsular surface (necrosis). There was a moderate amount of viscous clear fluid containing yellow, flocculent material in a branching linear pattern on the parietal surface of the lungs bilaterally (mucus, inflammatory exudate)

Histopathology:
Liver – scattered throughout the parenchyma, there are multiple, randomly-distributed patches in which the hepatic architecture is lost and the tissue replaced by amorphous eosinophilic material containing condensed and fragmented nuclei (necrosis). Associated with the regions of necrosis are large numbers of small (2-4µm), round to ovoid organisms, each with an eccentric nucleus (protozoal parasites), which are either free or, occasionally, within the cytoplasm of an hepatocyte. Besides the necrotic areas, there are also multiple, randomly-distributed mild to moderate infiltrates of primarily macrophages, with fewer lymphocytes.
Spleen – in multiple, coalescing patches of varying size, and affecting approximately 40% of the splenic parenchyma, the normal architecture is lost and replaced by eosinophilic material containing condensed and fragmented nuclei (necrosis). Myriad protozoal organisms as described above are present throughout the necrotic tissue.
Heart – occasionally through the myocardium, there are small to moderate clusters of protozoal parasites as described, with no clear evidence of a tissue response.
Lungs – scattered throughout the parenchyma, there are infrequent small clusters of protozoal parasites as described, with a moderate number of air capillary stromal cells having pyknotic nuclei (apoptosis, necrosis).

Final comment:
The penguin's death is attributable to a systemic inflammatory response, likely leading to a state of shock, arising from the extensive splenic and hepatic necrosis associated with an acute protozoal infection. Morphologically, the parasites are consistent with *Plasmodium* spp. as has been previously reported, though this diagnosis remains to be confirmed. No histological evidence of pleuritis was found.

Case 16: 12/524

Gross findings:
With a body weight of 1240g, flattened pectoral musculature and with prominent keel and femurs, the bird had a body condition score of 2.5/5. At 7.6% of bodyweight (reference range 2.30 – 5.34), and extending 20mm beyond the xiphoid process, the liver was moderately enlarged. Randomly scattered over the capsular surface and throughout the parenchyma there were multiple 1–2mm

flat, off-white areas of discolouration (necrosis). Weighing 6.12g and, across the short axis, spanning 3.5 times the diameter of adjacent small intestine, the spleen was markedly enlarged. Also, there were speckled 0.5-3mm areas of tan discolouration on the capsular surface (necrosis).

Cytology:
Impression smears of liver and spleen were stained with Wright's-Giemsa stain. The smears are highly cellular and contain, respectively, many erythrocytes and splenic and hepatic parenchymal cells against a dense, proteinaceous background. At the edges of the hepatic smear, numerous 3-5µm, oval bodies with a thin outer membrane, palely basophilic cytoplasm and a sharply demarcated, irregular, brightly eosinophilic nucleus are seen (apicomplexan parasites).

Histopathology:
By request of the submitter, histological examination of the moderately decomposed tissues was not conducted.

Final comment:
In view of the moderately advanced decomposition of the penguin, an abbreviated post mortem examination was requested and performed. However, given the gross similarities of this case with the numerous penguins diagnosed in the past 10 months with Avian Malaria, and given the protozoan parasites seen on cytological examination of the liver impression smear, it is reasonable to conclude that this penguin's death was attributable to a systemic inflammatory response, likely leading to a state of shock, arising from splenic and hepatic necrosis associated with an acute protozoal infection. Morphologically, the parasites are consistent with *Plasmodium* spp. as has been previously reported, though this diagnosis remains to be confirmed.

Case 17: 13/081

Gross findings:
With a bodyweight of 1320g, good soft tissue coverage of bony prominences and slightly convex pectoral muscles, a body condition score of 3/5 was given. Scattered over the capsular surface and throughout the parenchyma on the cut surface of the liver, there were multiple, circular, yellow-white spots of up to 2mm diameter (necrosis). At 5.2% of bodyweight, the liver is at the upper limit of normal size. At 5.68g, the spleen was markedly enlarged and scattered across its capsular surface were multiple circular yellow-white spots of up to 1mm diameter, similar to those seen in the liver (necrosis).

Histopathology:
Liver – the tissue is markedly autolysed, with virtually no cellular detail discernible. At scattered points throughout the tissue, there are clusters of intact small (1-2µm) ovoid nucleated organisms consistent with apicomplexan protozoal parasites within small foci of hepatocellular necrosis.

Spleen – though all cellular elements exhibit signs of degeneration (cytoplasmic swelling, condensed or distorted nuclei), there are multiple foci scattered randomly throughout the parenchyma in which all cellular detail is lost (necrosis). In many of the necrotic foci, there are clusters of ovoid organisms as seen in the liver, both free within the necrotic debris and in the cytoplasm of macrophages.

Final comment:
Marked autolysis complicates a thorough histological evaluation of this bird's tissues, though the gross findings and the presence of apicomplexan parasites in the necrotic centres in the spleen and in scattered locations in the liver effectively confirm a diagnosis of haemoproteosis, an infection which has been documented frequently in birds from this population in the last couple of years.

Case 18: 13/670

Gross findings:
With a bodyweight of 1090g and flat pectoral muscles, a body condition score of 3/5 was given.
Liver – there were occasional, poorly-defined 1-2mm diameter roughly circular patches of pale brown discolouration on the capsular surface (necrosis) and at 59.8g the liver was moderately enlarged. At 4.7g, the spleen was markedly and uniformly enlarged, with multiple, pale-red, 0.5-1mm diameter spots on the capsular surface (splenomegaly and necrosis).

Histopathology:
Liver – affecting the entirety of the liver, the cells are swollen and eosinophilic with a loss of all nuclear detail, though the general architectural arrangement of the tissue is retained (post-mortem autolysis). Randomly distributed throughout the tissue, there are patches in which all cellular detail is lost (antemortem necrosis). Associated with these patches are numerous clusters of intact small (1-2µm) ovoid nucleated organisms consistent with apicomplexan protozoal parasites (presumed *Haemoproteus* sp.)
Spleen – there are multiple foci scattered randomly throughout the parenchyma in which all cellular detail is lost (necrosis). In these necrotic foci, there are clusters of ovoid organisms as seen in the liver, both free within the necrotic debris and in the cytoplasm of macrophages.

Final Comment:
The gross and histological findings effectively confirm the diagnosis of haemoproteosis, an infection that has been seen intermittently in this population since late 2011.

Other

In one additional case, 13/083, there was histological evidence of mononuclear cell hepatitis with rare ovoid bodies consistent with protozoal organisms and a mildly enlarged spleen. In this case the bird had become trapped in wire mesh and had been found dead. Gross and histopathological

examination could not ascertain the cause of death, though haemoproteosis was considered unlikely.

			Parasites detected				
Case #	Hepatomegaly	Splenomegaly	Liver	Spleen	Heart	Lung	Other
1 - 06/1172		✔	✔	✔		✔	Parasites in adipose tissue, pericardial artery
2 - 08/1075			✔	✔	✔	✔	Bile duct distension
3 - 11/484			✔				No spleen sample or weight recorded
4 - 11/624		✔	✔	✔		✔	
5 - 11/626	✔	✔	✔	✔			
6 - 11/627	✔		✔	✔			
7 - 11/628	✔		✔	✔	✔		
8 - 12/020			✔	✔			
9 - 12/021			✔	✔			
10 - 12/338			✔	✔			Newcastle disease virus, suspect +ve
11 - 12/362	✔	✔	✔	✔	✔		
12 - 12/375	✔	✔	✔	✔	✔	✔	Parasites in air sacs, pericardium. Histiocytic myositis (no parasites in the muscle lesions)
13 - 12/385	✔	✔	✔	✔	✔		
14 - 12/473	✔	✔	✔	✔	✔		
15 - 12/517	✔	✔	✔	✔	✔	✔	
16 - 12/524	✔	✔	✔				No histology, parasites detected on cytology
17 - 13/081		✔	✔	✔			
18 - 13/670		✔	✔	✔			

Summary

The common features in these cases are birds in good body condition with livers tending to be large, moderately to markedly enlarged spleens, multiple, randomly-distributed areas of necrosis in the liver and spleen with small protozoal organisms in the necrotic lesions in these two tissues. Other tissues in which the organisms are reasonably often detected include the heart (7 of 18) and lungs (4 of 18) and much less commonly adipose tissue (1 of 18) and pericardium (2 of 18). Figures 3 and 4 below show typical histopathological findings, and figure 5 shows typical cytological findings from several cases with the protozoan organisms clearly visible in figures 2 and 3.

There are few associated comorbidities. One penguin (case 10 - 12/338) was suspect positive for Newcastle disease virus by PCR to detect antigen. One penguin (case 12 - 12/375) had a histiocytic myositis, though without evidence of parasites in this tissue. In one case (case 16 – 12/524), the diagnosis was made without histology, but on the gross changes and cytological evidence. Note that in cases 17 and 18, the pathologist's final comment indicated a diagnosis of haemoproteosis; these cases were reported after early Polymerase Chain Reaction results had identified infection with a novel *Haemoproteus* spp. parasite in some penguins from Penguin Island; this will be discussed more fully in chapter 5.

Protozoan parasites are readily-detectable by light microscopy if a directed effort is made to find them, though they could be overlooked if a protozoal infection is not suspected, or if cellular debris in necrotic tissues makes them difficult to identify. Note, though, that the resolution of external morphology and internal features at the scale of these organisms means that identification beyond the level of phylum (i.e., Apicomplexa) requires additional techniques such as Polymerase Chain Reaction (PCR), In-situ Hybridisation (ISH) or electron microscopy. While the majority of the reports compiled here suggest that the infection is likely to be due to *Plasmodium* spp. parasites, this suggestion was made on the basis that captive penguins have often been reported to be susceptible to this infection with similar post mortem findings. Clear evidence of erythrocyte infection is lacking in all of these cases. On morphological evidence other Haemospororida such as *Haemoproteus* and *Leucocytozoon*, and other Apicomplexa such as *Toxoplasma* and *Sarcocystis* must also be considered as potential agents of disease.

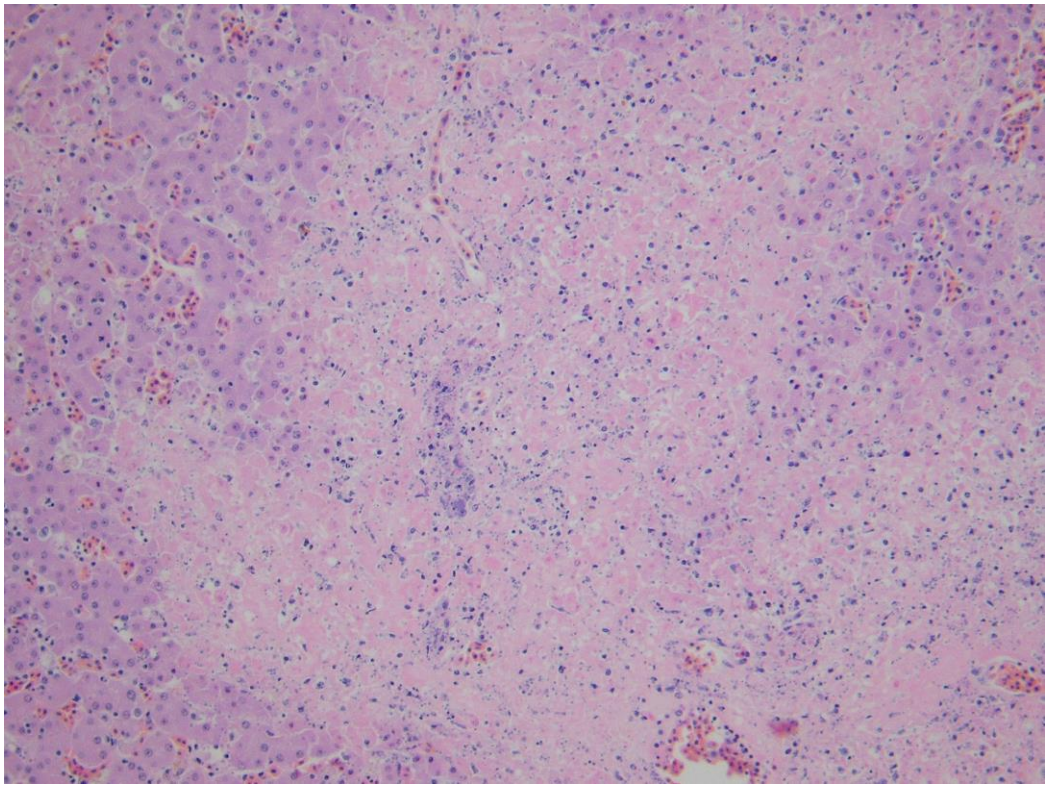


Figure 3 – case 14, liver, necrotic focus (the area of relative pallor, within which numerous organisms were identifiable)(5µm section, Haematoxylin and Eosin stain, 200x)

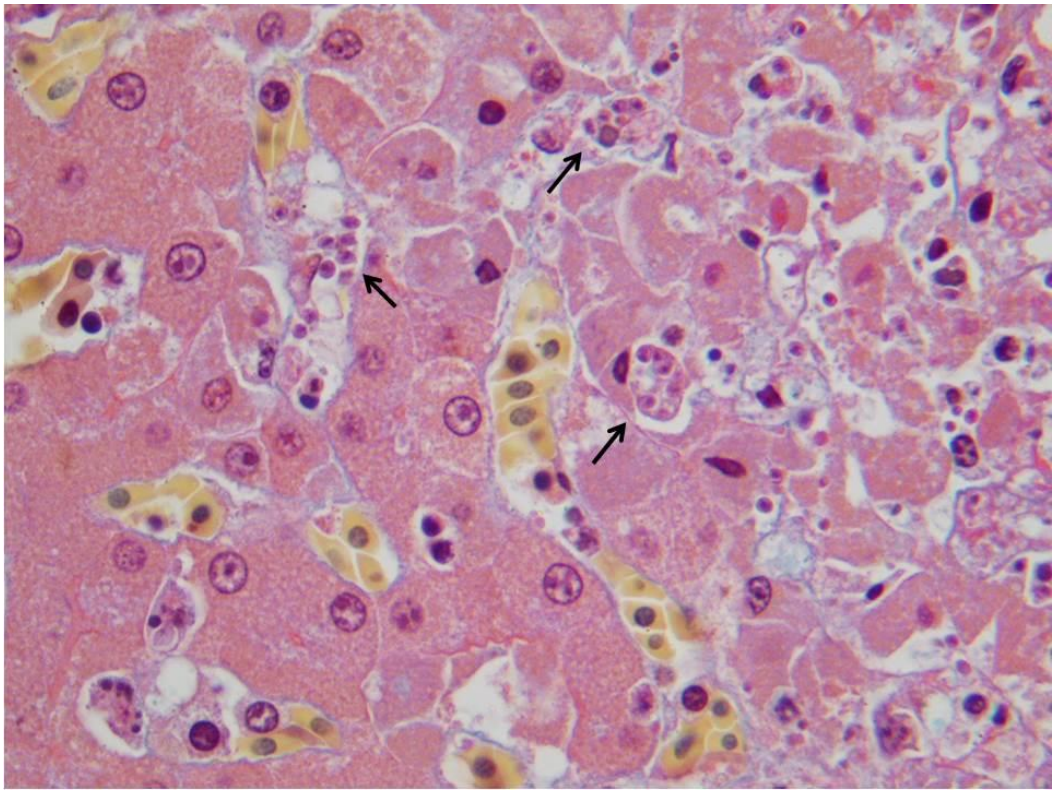


Figure 4 – case 14, liver, intact and necrotic hepatocytes and numerous protozoa (arrows), free and within cysts

(2µm section, Martius Scarlet Blue stain, 1000x)

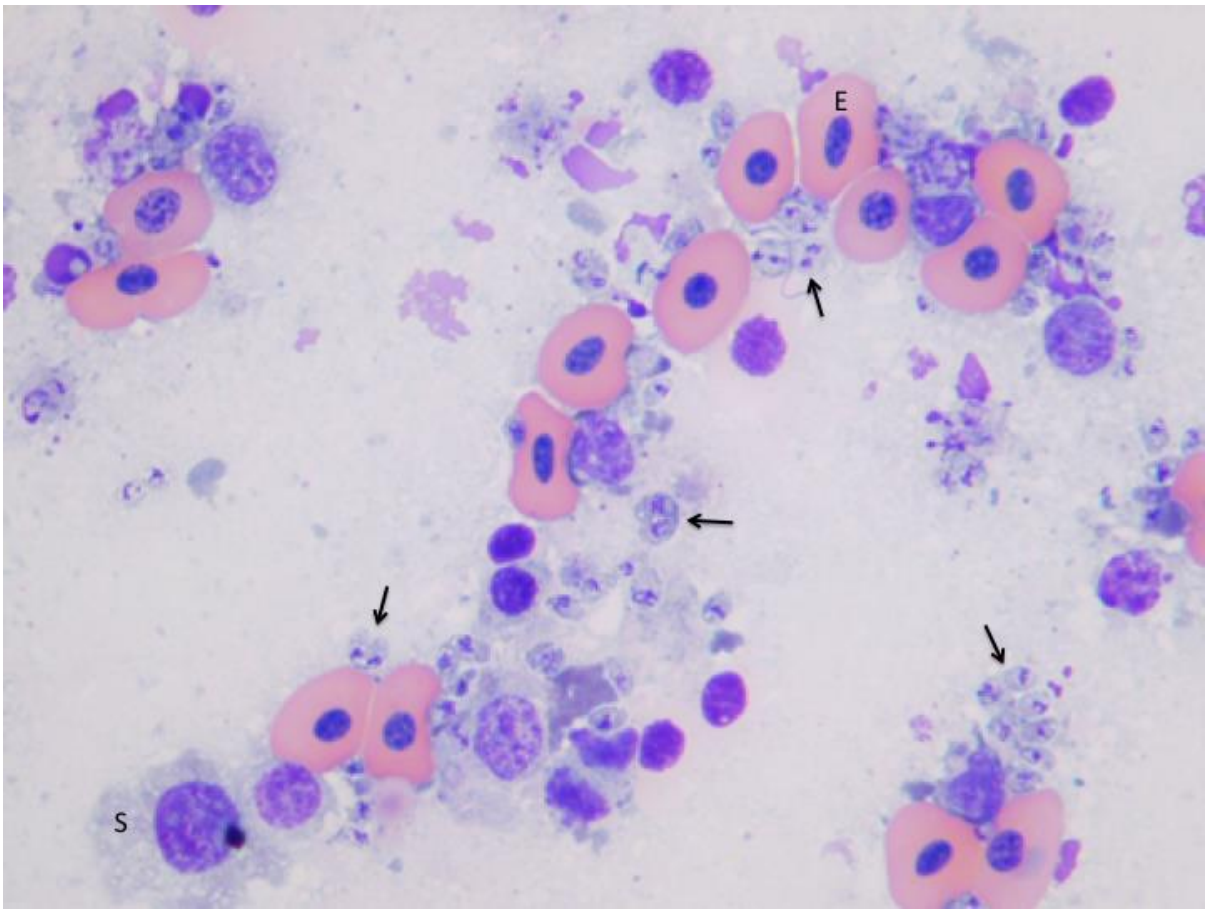


Figure 5 – case 11, splenic impression smear, erythrocytes (E), splenic stromal cells (S) and numerous protozoa (arrows)(Wright's Giemsa stain, 1000x, case 11 – 12/362)

3. Fieldwork

Introduction

From the post mortem examinations, it had been established that a disease existed in this population of Little Penguins in which the key post mortem findings of mild to moderate hepatomegaly, marked splenomegaly and multifocal hepatic and splenic necrosis were associated with the presence of intralesional protozoal parasites in those tissues, and occasionally in erythrocytes. As previously discussed, these observations were suggestive of an infection with a haemoparasite in which the tissue phase resulted in such extensive and acute tissue necrosis as to induce a state of shock.

Similar gross and microscopic lesions had not been observed in other penguins presented for post mortem. It appeared that, at least in the sample population we had, exposure to this infection was, or at least could be, rapidly fatal. Working hypotheses were developed which stated that:

1. The penguins were immunologically naïve to the infection, and thus were aberrant hosts in the life cycle of this parasite
2. Another bird species present on Penguin Island, whether permanently or intermittently, was the definitive host for this parasite
3. The penguins had recently become exposed to this new infection either because there was a greater prevalence of parasitaemia in the definitive host, or because there was a new competent invertebrate vector by which the parasite could be transmitted to other hosts

We wished to investigate whether exposure to the parasite was invariably fatal for the penguins, or whether some that had been infected had survived to become parasitaemic. Also, we wished to investigate which other bird species that used Penguin Island might be carriers of the disease. Besides the Little Penguin, bird species that breed on Penguin Island include Bridled Terns (*Sterna anaethetus*), Caspian Terns (*Hydroprogne caspia)*, Silvereyes (*Zosterops lateralis*), Singing Honeyeaters (*Lichenostomus virescens),* Silver Gulls (*Chroicocephalus novaehollandiae*), and Buff-banded Rails (*Gallirallus philippensis)*[67]. Other birds that make visits to the island include Welcome Swallows (*Hirundo neoxena*), Willy Wagtails (*Rhipidura leucophrys*), Ravens (*Corvus coronoides*), Australian Kestrels (*Falco cenchroides*), Little Eagles (*Hieraaetus morphnoides*), Swamp Harriers (*Circus approximans*), Barn Owls (*Tyto alba*), Boobook Owls (*Ninox novaeseelandiae*) and Pelicans

(*Pelecanus conspicillatus*)[67]. Conceivably, any one of these species may represent the host of the parasite that came to be infectious to the penguins.

In order to achieve the above stated aims, we decided to collect peripheral blood samples from the penguins and selected other species and to make blood smears which could be assessed for the postulated erythrocytic stages of the parasite. Examination of blood smears is a simple and easily-performed technique which is routinely used to diagnose *Plasmodium* infections (malaria) in humans.

Two avian species which breed on Penguin Island were prioritised as candidates for blood sampling, the Bridled Tern and the Caspian Tern. The Bridled Tern is a seabird which breeds on Penguin Island in the period from September to May. During the winters, the terns migrate to the north-west Sulawesi Sea between the Indonesian and Philippine archipelagos[26]. In this period, the birds are likely to be exposed to vector-borne infectious organisms which they may then carry back to Penguin Island for the next breeding season.

The Caspian Tern is another migratory seabird which breeds in the Perth metropolitan area from July to November[57]. Local breeding and roosting sites include Rottnest, Carnac, Bird, Gull and Seal islands, as well as Penguin Island, and their nest sites vary from year to year[57]. Outside of the breeding season, they migrate to estuarine habitats, which presumably exposes them to a variety of invertebrate vectors.

Materials and methods

Blood collection and blood smear preparation

Penguins

An online sample size calculator (http://sampsize.sourceforge.net/iface/index.html#prev) was used to determine the appropriate sample size for a disease prevalence survey, using the following parameters:

1. Precision - 10%
2. Prevalence estimate - 17% (based on the 9 of 54 penguins presenting for post mortem which had had evidence of protozoan infection)

3. Population size - 950 (current Penguin Island population estimate)
4. Confidence interval - 95%

The recommended sample size derived from the calculation was 52. Based on this calculation, it was proposed to collect samples from 52 penguins in 2012 and to repeat the collection in 2013, making a total of 104 samples across the two years.

Prior to the commencement of fieldwork, the proposed capture and blood collection protocols were approved by the Murdoch University Animal Ethics Committee (Permit No. RW2510/12). All bird handling and blood collection procedures were performed by one of the named operators on the Animal Ethics Permit or, on one occasion, by a veterinarian and Animal Welfare Officer from the Animal Ethics Committee (Dr Cree Monaghan) who was in attendance to monitor the procedures and offer guidance. Seven separate visits were made to Penguin Island (32° 18' S, 115° 41' E) to collect blood from penguins; four of these visits were made during the day on 26/07/12 (12 samples), 21/08/12 (14 samples), 14/09/12 (17 samples) and 15/10/12 (11 samples), and three were overnight visits on 24/11/12 (7 samples), 06/12/12 (15 samples) and 29/09/13 (17 samples).
A total of 94 penguin blood smears were made and examined. Of these, several were repeat blood smears from previously sampled animals. Penguin #208116 was sampled on two occasions (21/08/12 and 29/09/13), as were penguin #208357 (24/11/12 and 29/09/13), penguin #211387 (21/08/12 and 14/09/12), penguin #215672 (14/09/12 and 15/10/12), penguin #264455 (26/07/12 and 21/08/12), penguin #458030 (14/09/12 and 06/12/12), penguin #460705 (21/08/12 and 15/10/12), and penguin #461519 (06/12/12 and 29/09/13). Penguin #213543 was sampled on three occasions (26/07/12, 21/08/12 and 14/09/12), as was penguin #399204 (21/08/12, 14/09/12 and 15/10/12). One penguin, #252549, was sampled on four occasions (26/07/12, 21/08/12, 15/10/12 and 24/11/12). Thus, the 94 smears represent samples from 79 individual penguins, and they were collected from the austral winter to summer of 2012 and in the early austral spring of 2013. Sampling during these periods was intended to detect infections in different seasons, in the event that there was a seasonal component to exposure, for example, increased vector numbers during warmer weather. Also, the majority of cases seen at post mortem had been presented in late austral spring and summer, so it was considered important to include these seasons in the collection periods.

During the daytime collections, penguins were captured during checks of the nestboxes which are scattered around Penguin Island. Any penguin present in a nestbox was gently removed (providing it

did not have very young chicks, in which case it was left otherwise undisturbed). Each penguin was weighed and scanned for a microchip if no flipper band was present. Blood collection was performed immediately, and as near to the nest box as possible, then the penguin was either returned to the nest box straight away, or placed in a small enclosure (specifically, a plastic cat carrier) for no longer than 15 minutes for the purpose of collecting a faecal sample (for a separate study concurrently being conducted by Dr Belinda Cannell). While in the cat carrier, the penguins were attended at all times until they were returned to their nestboxes.

For night collections, the penguins were captured at or shortly after dusk when they returned to the island from foraging at sea. By habit, the penguins make landfall at specific beaches that are closest to their nests. To corral the birds, the methodology described in Cannell *et al* (2011) was followed. Briefly, low fences constructed from rolls of shadecloth and plastic lattice were erected at various landing sites at two of the major arrival beaches for the penguins - the southern and eastern beaches. The fences were oriented so as to channel any penguins coming ashore at those sites into small corrals which could then be closed. The penguins were then removed to holding pens consisting of large, open-bottomed cardboard boxes placed out of sight of the corral so as not to deter other penguins arriving. The birds were collected over a period of 30-60 minutes. At the end of this time, they were removed one at a time for weighing, scanning for microchips (or recording of flipper band ID) and blood collection; any birds without a microchip or flipper band were microchipped at this time. After sampling, the birds were released close to the beach at which they had arrived.

Peripheral blood was collected from the metatarsal vein on the dorsal aspect of one or the other foot. During the first four collection trips, the birds were restrained by hand and were partially wrapped in a calico bag to cover their heads and upper bodies. After gaining approval from the Research Ethics and Integrity office, the penguins were subsequently restrained using an equine knee/skid boot which consists of a sturdy yet soft, approximately cylindrical support, open at each end and along one side where it can be secured by three Velcro straps. The penguins were placed lengthwise in this device with their backs uppermost, and the straps were then secured to form an open-ended tube with the penguin safely encased within (Fig 1). From this position, a foot could be grasped, externalised and restrained. After swabbing with methylated spirit, a 23 or 25 gauge butterfly needle was inserted into the metatarsal vein, and up to 1mL of blood was collected into a 1 or 2mL syringe with slight negative pressure. After sampling, a fresh dry swab was applied to the venipuncture site with gentle pressure until there was no evident bleeding. No animals were injured

during capture or sampling, and any signs of mild distress (e.g., vocalisation) ceased on the birds' release.

Figure 1 – equine knee/skid boot used for penguin restraint (1:1 scale model penguin in situ)

In each case, a standard thin blood smear was made at the time of blood collection and the slide was air dried and stored for later staining; any remaining blood was expelled into a labelled 2mL plastic blood tube containing potassium EDTA, and this tube was retained until the prepared smears had been stained in case additional smears were required. In many cases, only enough blood to make a single blood smear was obtained.

Within five days of smear preparation, the slides were stained with Wright's Giemsa stain on an automatic stainer, then manually cover-slipped.

To assess each slide for the presence of parasites, a minimum of 20 oil immersion fields were examined. Red cells in the monolayer were observed for the presence of intracytoplasmic organisms.

Bridled Terns

Blood collection and its protocols were approved by the Murdoch University Animal Ethics Committee (Permit No. RW2510/12). Bridled Terns (*Sterna anaethetus*) were captured at night during two visits to the island on 24/11/12 (21 samples) and 02/03/13 (31 samples, no repeats). To capture a tern, one of two registered bird banders (Dr Nic Dunlop or Ms Sandy McNeill) would shine the light from a headlamp towards the bird, and then place a soft net over the bird while it sat immobile. The terns were restrained by holding their wings against their bodies, which is easily accomplished with a single hand in this small species. Birds were checked for leg bands, the number on the bands was identified, and those without a leg band were then banded.

Blood was collected from the either the leg vein (where visible) as it ran over the lateral tarsus, or, more frequently, from the wing vein. The birds were held in dorsal recumbency with a wing, usually the right, held in extension, and the wing vein was identified as it ran over the medial aspect of the humerus just proximal to the elbow. After swabbing with methylated spirit, a 25 gauge needle attached to a 1mL syringe was inserted into the vein and up to 0.1mL of blood was collected into the syringe. A blood smear was made at the time of blood collection and air dried for later staining. After wing venipuncture, a small (typically 5 x 5mm) square of Kaltostat dressing (ConvaTec Inc., Skillman, NJ, USA) was applied to the puncture site and held in place for approximately 30 seconds. Kaltostat is a calcium alginate dressing which assists in the formation of a haemostatic plug at bleeding wound sites. All birds were able to fly without difficulty on release.

Within five days of smear preparation, the slides were stained with Wright's Giemsa stain on an automatic stainer, then manually cover-slipped.

To assess each slide for the presence of parasites, a minimum of 20 oil immersion fields were examined. Red cells in the monolayer were observed for the presence of intracytoplasmic organisms.

Caspian Terns

Two visits were made to Penguin Island with the intention of collecting blood samples from Caspian Terns (*Hydroprogne caspia*). On both occasions, inclement weather reduced the time available for attempting to capture the birds. Additionally, these attempts were made relatively late in the Caspian Tern's breeding season, and the birds had become wary of the traps used to capture them

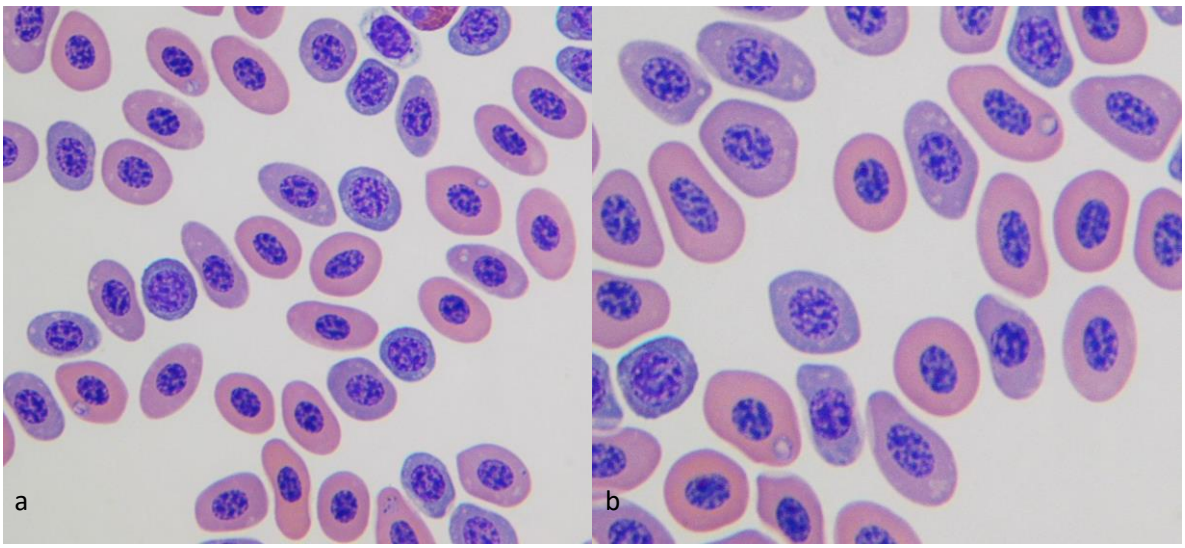
(in many instances they had previously been trapped for banding). Ultimately, no birds were trapped on either occasion, and no samples could be collected from this species.

Results

Penguins

Intraerythrocytic parasites were detected in one blood smear. The smear was collected from penguin #215057, which was captured on the island's south beach on 06/12/12 (i.e., during the austral summer). This penguin was one of a group captured and held for approximately 30 minutes before sampling. It was in excellent body condition, with a body mass of 1850g. This is well above the average mass of males on Penguin Island, which is 1348 ± 200g (n=118) (Cannell, unpublished data), and it was most likely that this bird was amassing fat in preparation for its annual moult (Cannell pers. comm.) No bird in this group showed signs of being systemically unwell (e.g., lethargy, ataxia, respiratory distress) at the time of capture, blood collection or release.

The blood smear for this penguin showed evidence of a regenerative erythrocyte response, with abundant immature erythrocytes identifiable by their morphology (large, round nuclei, relatively round cells with basophilic cytoplasm). In the cytoplasm of a small percentage of cells there were one or more 1-3µm long ovoid bodies with clear cytoplasm and an apical nucleus (see Fig 2). These bodies are consistent with the blood stages of apicomplexan parasites, for example, merozoites or early gametocytes of *Haemoproteus* or *Plasmodium*, or merozoites or sporozoites of *Babesia*. The evidence of erythrocyte regeneration suggests a resolving anaemia, presumably due to haemolysis in response to the erythrocytic parasites.



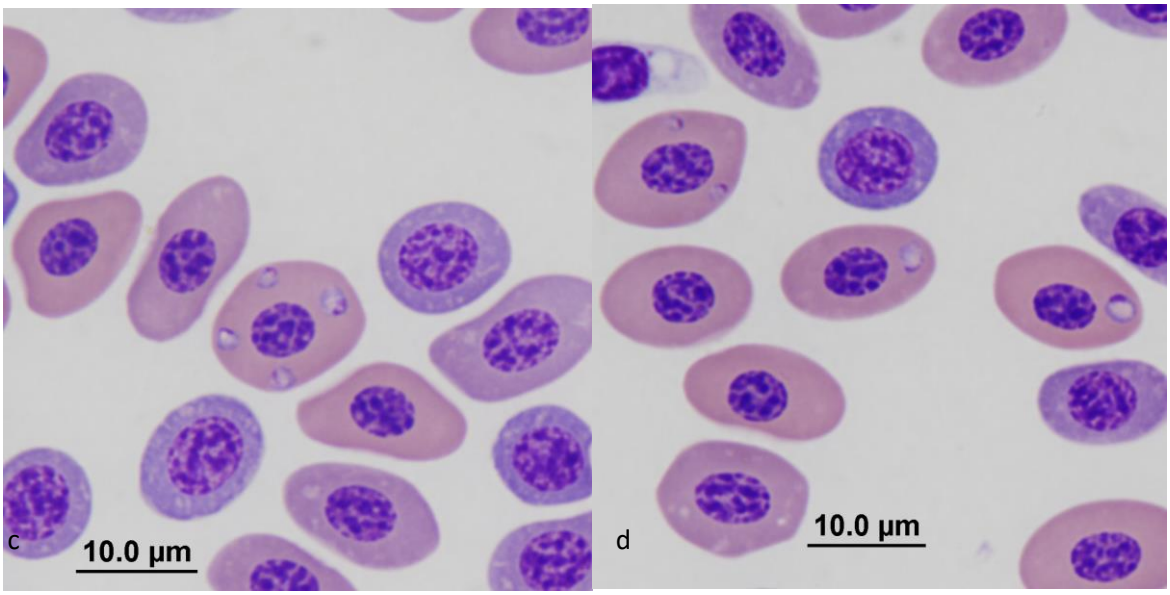


Figure 2, a to d: protozoan parasites in the cytoplasm of erythrocytes (penguin #215057, collected 06/12/2012) (Wright's Giemsa stain, 1000x)

Bridled Terns

No parasites were detected in any blood smears from Bridled Terns.

Summary

A confident estimate of the prevalence of haemoparasite infection in Penguin Island Little Penguins cannot be made on the evidence of a single positive bird in 94 samples from 79 individuals. However, it appears that the overall prevalence of parasitaemia in both Little Penguins and Bridled Terns is low.

The morphology of the parasites seen in penguin #215057 does not allow identification to the level of genus. While they are somewhat consistent with the gametocytes of *Haemoproteus* parasites, there are reasons to suspect that is not what they are. *Haemoproteus* gametocytes develop within 7-10 days to occupy most of the cytoplasm of the erythrocyte[6], and all of the parasites seen in this bird are of approximately the same size. Conceivably, a mass release of merozoites could lead to erythrocyte infection by a single, very early stage of parasites, though this must be considered improbable.

An alternative explanation is that the parasites in this smear are a haemoparasite such as the piroplasms *Babesia* or *Theileria*, which would be expected to show relatively little morphological variation throughout this life stage[40]. In support of this hypothesis is the evidence of red cell

regeneration on this smear; this may indicate recovery from a haemolytic anaemia, a typical consequence of piroplasm infection. Also, while it is not a haemoparasite (in that it does not ordinarily have a blood phase) *Toxoplasma* is another apicomplexan parasite with a comparable morphology; *Toxoplasma* does not infect the anucleate erythrocytes of mammals, though it can infect erythrocytes in avian hosts[22].

No haemoparasites were detected in the Bridled Tern smears. This may indicate an absence of haemoparasite infection, which is not unexpected in seabirds, or it may be due to seasonal variation in infection status, with infections occurring at other times of the year. Alternatively, it may be that there is a latent haemoparasite infection, and that parasitaemia is immunologically suppressed. This final possibility could be investigated with serology or with PCR to detect parasite antigen or antibodies. In any case, the hypothesis that Bridled Terns might be implicated as the source of the penguin infection can neither be confirmed nor refuted on this evidence.

4. Electron microscopy

Introduction

The very small size of the parasite infecting the penguins limits the extent of the morphological assessment that can be made from light microscopy. To improve the resolution and to allow evaluation of subcellular structures, representative samples were prepared for transmission electron microscopy. For this purpose, samples from 3 penguins were fixed in 5% glutaraldehyde at the time of post mortem. The samples were: liver and spleen from case 12/362; heart, spleen and liver from 12/375; spleen and liver from 12/385.

Materials and Methods

Samples measuring approximately 1 x 1 x 3mm were collected from fresh carcasses (i.e., not frozen) at the time of post mortem examination and fixed in glutaraldehyde at 4°C until required (approximately 4 months).

Processing for transmission electron microscopy was performed with the spleen and liver samples from case 12/362 only. This case was selected as it had a very high concentration of parasites visible by light microscopy, so it was considered that it offered the greatest probability of detecting the parasites in the very small tissue sections electron microscopy requires. The sample was processed in accordance with the standard protocols for the transmission electron microscope at Murdoch University.

The glutaraldehyde fixative was removed from the sample tube and replaced with Dalton's chrome osmic acid, then the sample was covered and allowed to fix for approximately 90 minutes. The sample was washed four times in 70% alcohol, followed by a 5 minute wash in 90% alcohol, then a 5 minute wash in 95% alcohol and then three washes in absolute ethanol for 3.5 minutes each. The sample was then washed in two changes of propylene oxide over 10 minutes before being left in a 60:40 solution of propylene oxide and epoxy resin for 1 hour. Finally, the sample was left on a rotator overnight.

The following day, the sample was placed in a vacuum chamber, at a negative pressure of approximately 30 inches of mercury, before it was embedded in fresh epoxy resin and baked at 60°C for 24 hours.

After baking, the resin blocks were trimmed to the margins of the sample, and then placed in a Reichert Om U3 ultramicrotome. A fresh glass knife, made on an LKB Bromma 7800 Knifemaker, was used for each block to cut thick sections at 1µm thickness. Sections were collected on a glass slide and stained with toluidine blue for initial light microscopic assessment to assess whether adequate numbers of host cells and parasites were present so as to be suitable to make thin sections. Thin sections were then made at 90nm thickness on a Reichert Ultracut E ultramicrotome, also using a freshly made knife. These sections were collected onto 200 mesh copper grids and stained with lead citrate and uranyl acetate.

Lead citrate stain was prepared by placing approximately 0.3g of lead citrate into a vial. The lead citrate was dissolved by adding 10mL of distilled water to the vial, followed by 0.1mL of 10N sodium hydroxide. The mixture was shaken, transferred to a centrifuge tube and centrifuged at 2,700rpm for 10 minutes. 10mL of stock saturated uranyl acetate solution was placed in a second centrifuge tube and centrifuged at the same time as the lead citrate solution.

To stain the sections, single drops of each solution were placed on a wax coated petri dish. The grids were first introduced to the base of the uranyl acetate drop, with the grid facing up, and allowed to stand for 7 minutes. They were then washed by dipping repeatedly in distilled water, and allowed to dry on filter paper. Once dry, the grids were placed on the top of the lead citrate drop, with the grid facing down, and allowed to float there for 4 minutes. The grids were washed again and allowed to dry on filter paper. Once stained, the grids were stored at room temperature until required.
Grids were examined using a Philips CM100 BioTwin Transmission Electron Microscope (Eindhoven, The Netherlands). Images were captured on photographic film and scanned to create digital copies.

Results

The following images were photographed from the electron microscopy sessions.

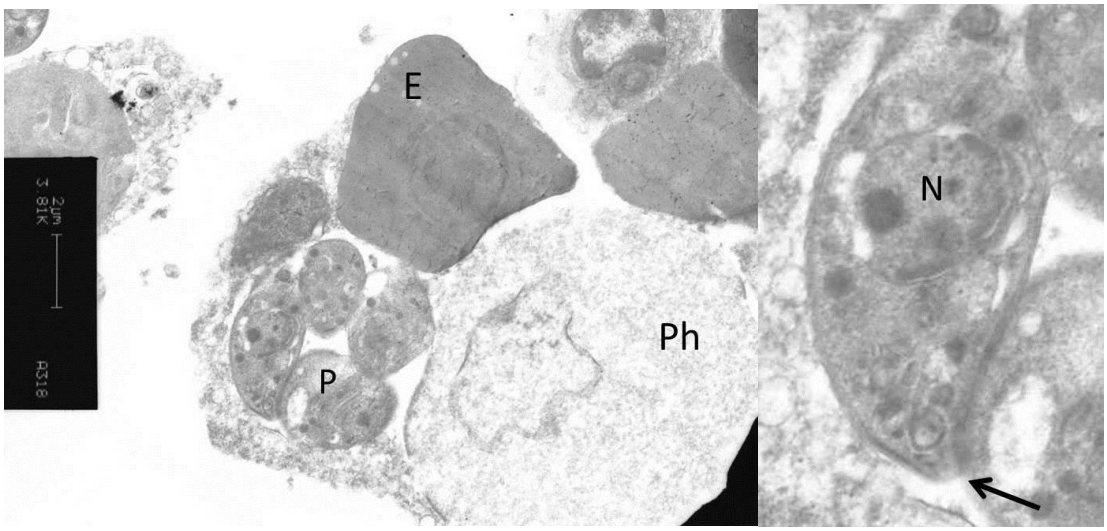


Figure 1 (and detail) – four parasites in a cyst within the cytoplasm of a host cell, spleen, x3810, bar = 2µm

Image left: E - erythrocyte, Ph - phagocyte, P - protozoa; image right (detail): N - nucleus

In Figure 1 there is a host erythrocyte (E) adjacent to a large, possibly phagocytic, cell (Ph). In the cytoplasm of the large cell, abutting its nucleus, there is a cluster of four protozoal organisms (one marked with a P), each approximately 1.5 to 2µm across. Though a membrane cannot be seen, the clear space surrounding the parasites is distinctly different from the large cell's cytoplasm, suggesting that the parasites are isolated in a vacuolar space. The left-most organism, which is enlarged in the detail image, is oriented to show the typical, slightly elongated apicomplexan morphology with a rounded base, a basal nucleus (N) and a cluster of organelles congregating at the tapered apex (arrow).

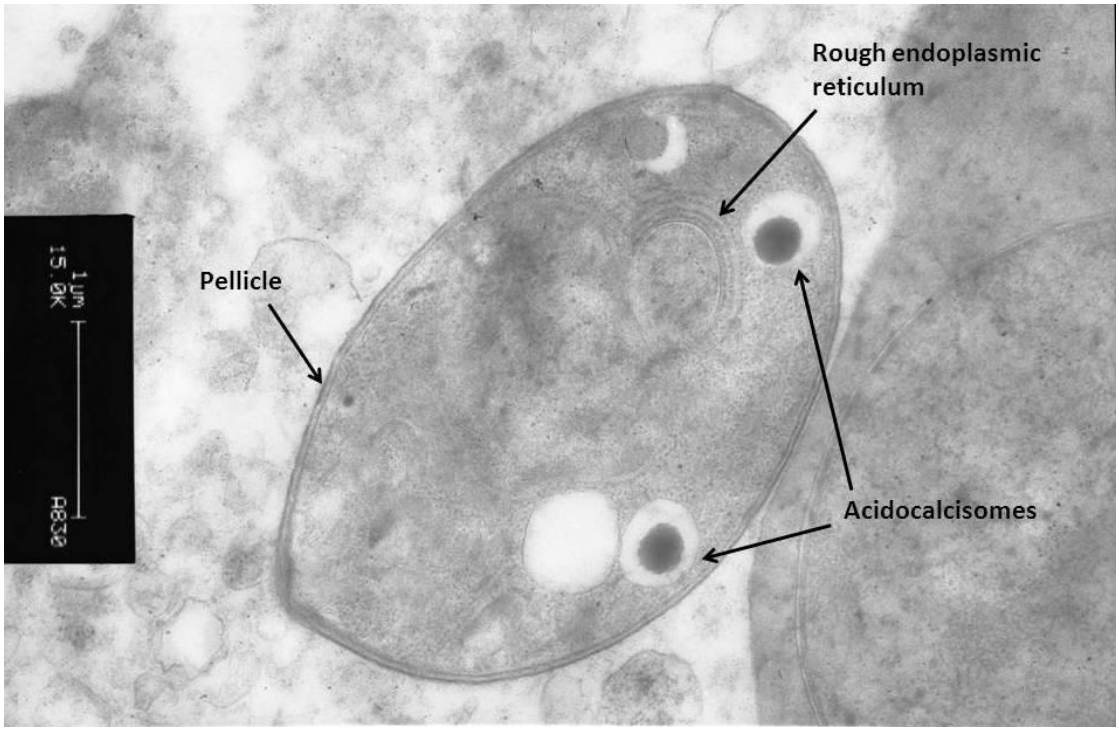


Figure 2 - Single extracellular parasite, spleen, x15000, bar = 1µm

Figure 2 demonstrates the pellicle, the outer wall of the organism, which consists of an outer and a continuous inner membrane[15]. Three acidocalcisomes and a crescent of rough endoplasmic reticulum are also present[15,58].

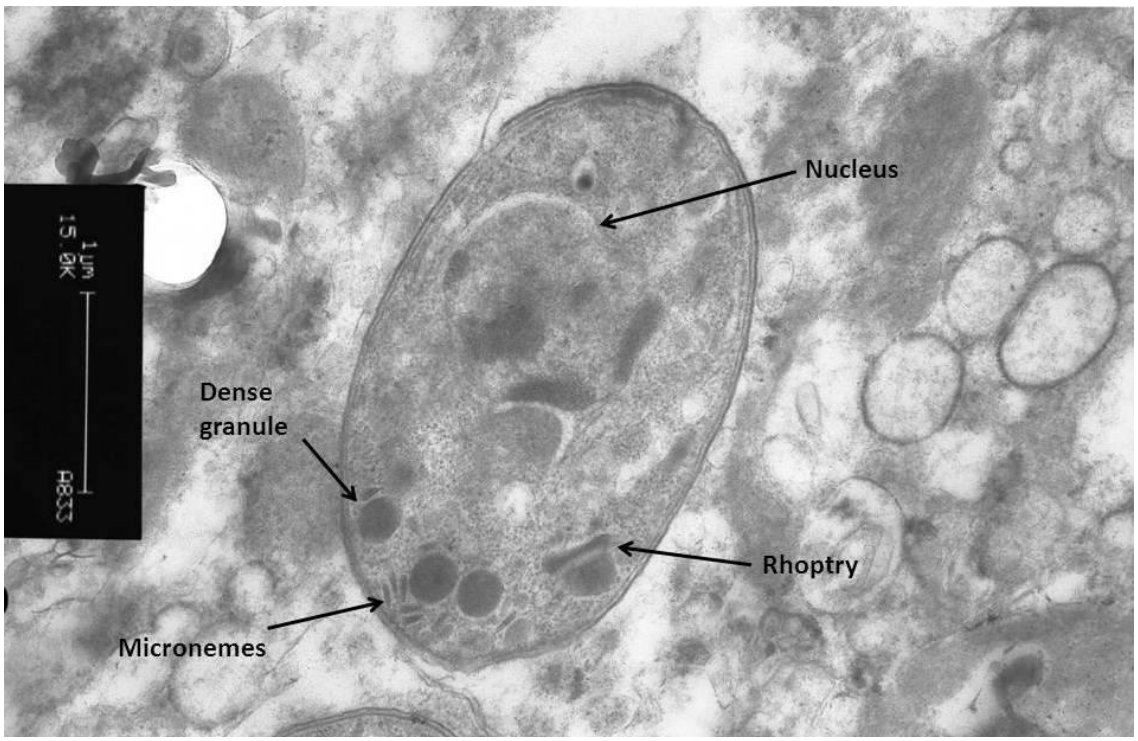


Figure 3 – apical complex, spleen, x15000, bar = 1µm

In Figure 3, several of the components of the apical complex are visible at the lower left hand side of the organism. The numerous short dark rods are micronemes[14]. Adjacent to these are several dense granules, and there is a larger elongate body next to one of the dense granules consistent with a rhoptry[15].

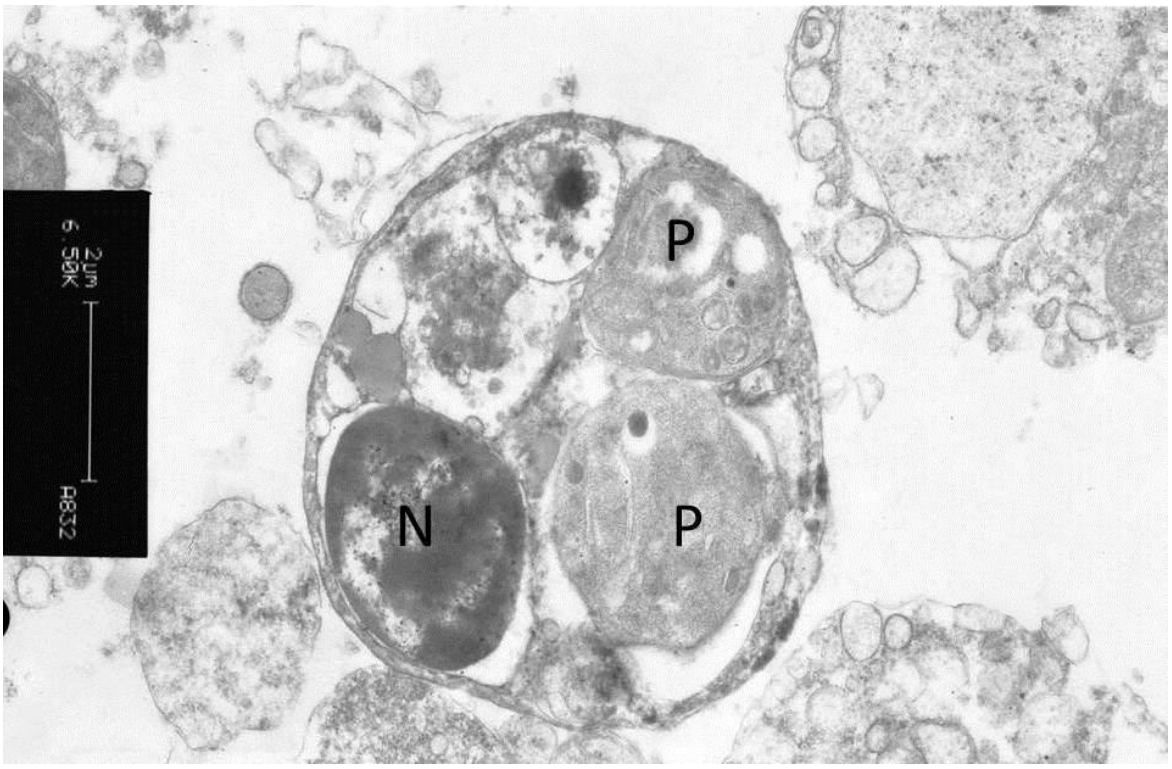


Figure 4 – two protozoa within a host cell, spleen, x6500, bar = 2µm.

P - parasites, N - nucleus (host cell)

In Figure 4 there are two distinct parasites (P) contained within the boundaries of another cell, which appears to be a host cell with a large, electron dense nucleus (N).

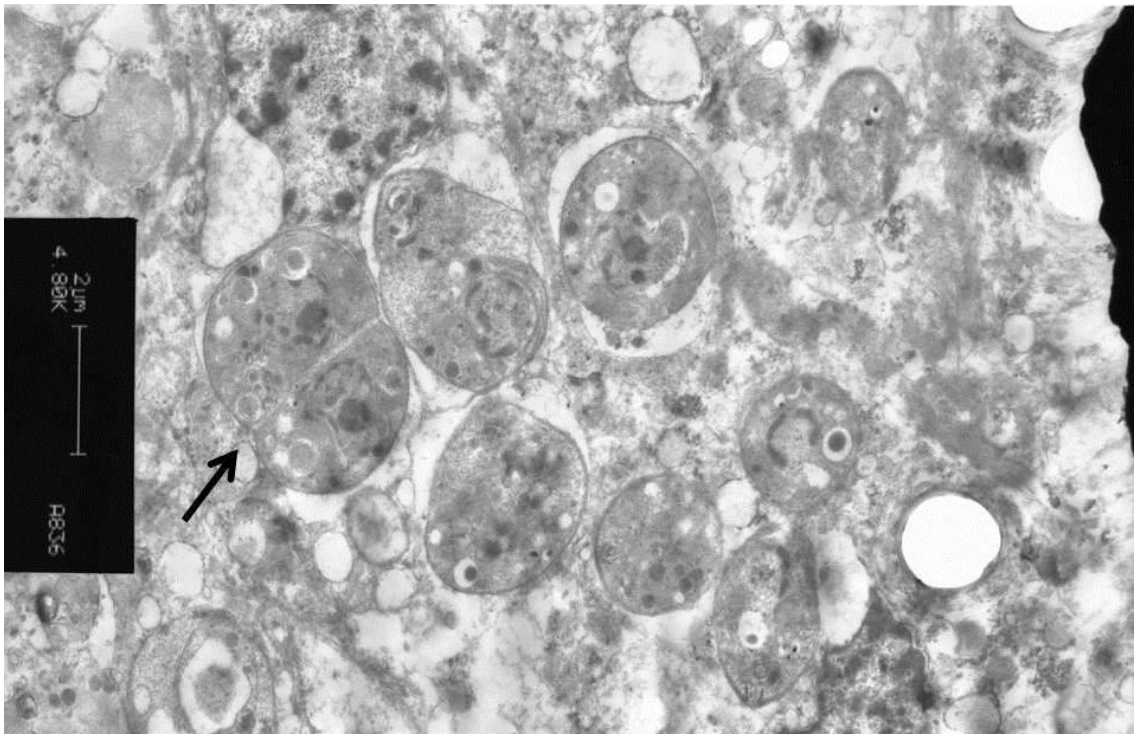


Figure 5 – a cluster of extracellular protozoa, spleen, x4800, bar = 2µm

Arrow - dividing protozoan

In Figure 5, numerous protozoa, one of which is dividing (indicated by arrow) are clustered in a background of amorphous material, presumed to be cellular debris from necrotic host tissues.

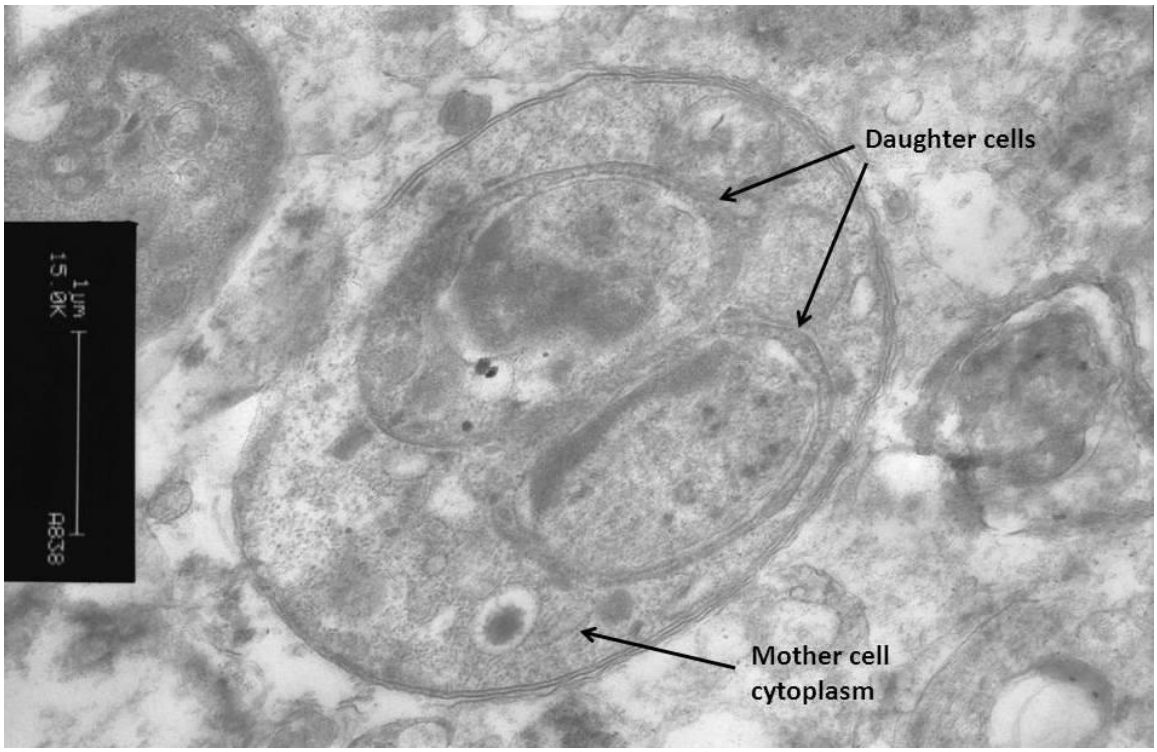


Figure 6 – early division, spleen, x15000, bar = 1µm

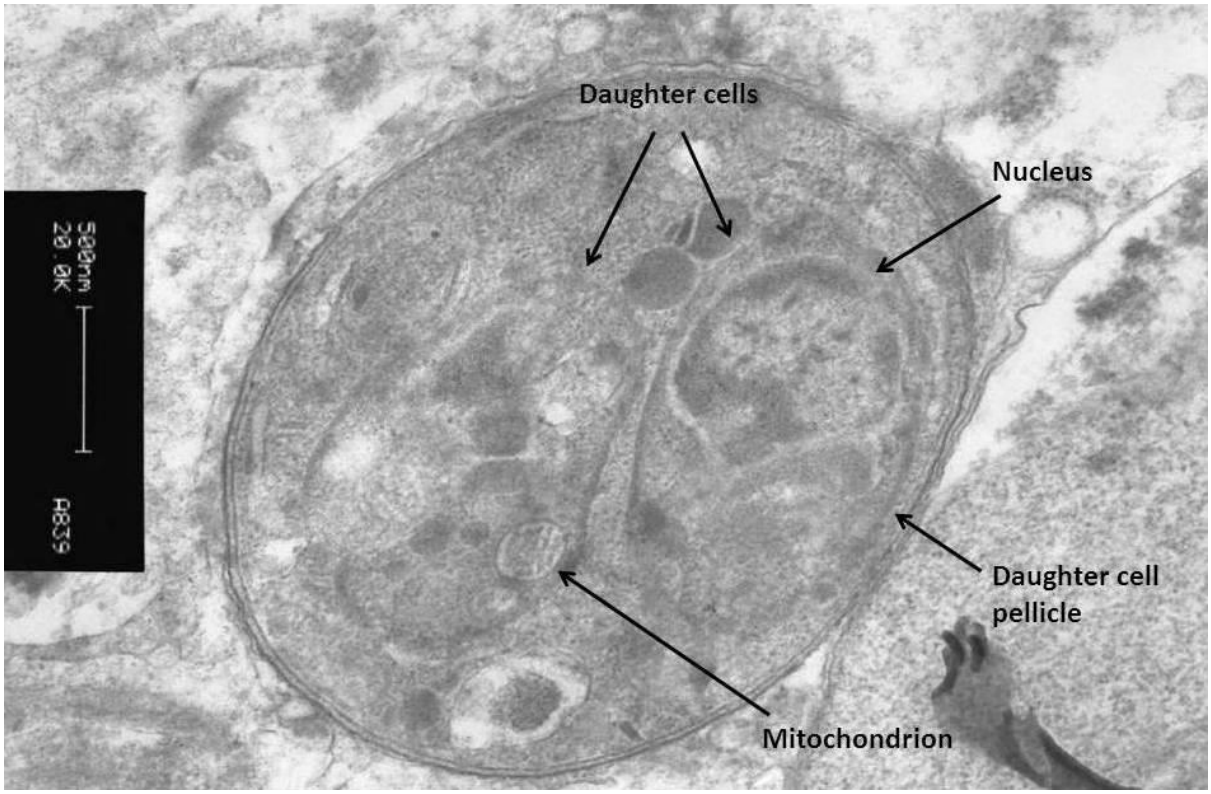


Figure 7 – early division, spleen, x20000, bar = 500nm

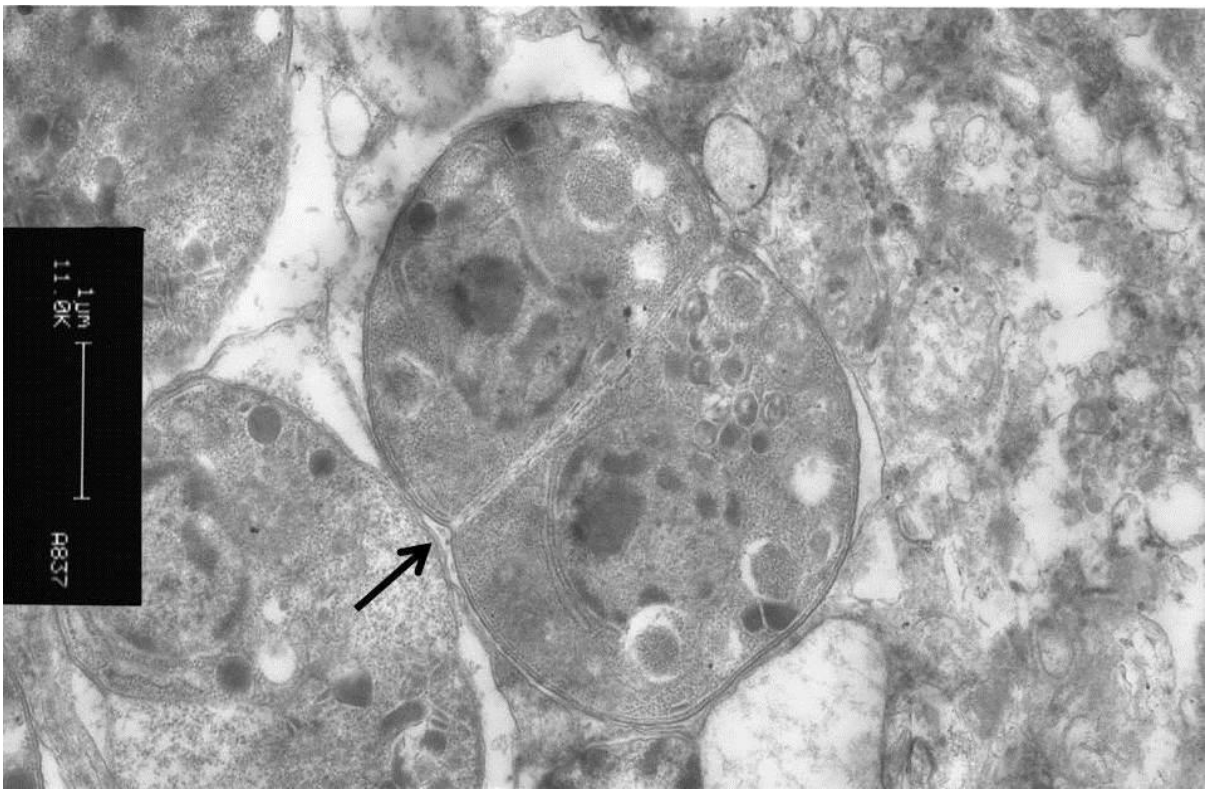


Figure 8 – late division, spleen, x11000, bar = 2µm

Arrow – membrane dissecting between dividing parasite cells

In figures 6 to 8, two distinct cells can be seen enclosed within a single pellicle. In figures 6 and 7, there are two nuclei enclosed in nascent pellicles with little cytoplasm and few organelles. The majority of the cytoplasm belongs to the mother cell. In figure 8, there are 2 virtually complete cells contained within a single pellicle, with a membrane dissecting between them (arrow).

Discussion

The identifiable ultrastructural features of this protozoan parasite include a pellicle, acidocalcisomes, dense granules, micronemes, rhoptries, and reproduction by a process of binary fission.
A pellicle is the name given to the plasma membrane of a protozoan organism when it is accompanied by an additional, continuous underlying membrane[15]. The pellicle is characteristic of the motile stages of sporozoan and ciliate parasites[15].

Acidocalcisomes are intracellular calcium stores that appear as round, clear spaces in the cytoplasm, often, though not invariably, containing a smaller, concentric or eccentric electron dense body. Acidocalcisomes are non-specific organelles which are highly conserved across a very broad range of species from bacteria to humans[15].

Dense granules are spherical, electron dense bodies that may occur anywhere in the cytoplasm of apicomplexan parasites. Their function is incompletely characterised, but some identified proteins have been associated with virulence[40].

Micronemes are small, elongate, electron dense secretory organelles that are confined to the apical segment of the parasite. These organelles are characteristic of apicomplexans and are involved in parasite attachment to host cells[40].

Rhoptries are another component of the apical complex. These secretory organelles are also incompletely characterised, though there is evidence that they are involved in the penetration of host cells and in the establishment of the parasitophorous vacuole in which parasites establish and divide in the host cell[40].

Considered together, the ultrastructural features of the protozoan parasite in the spleen of penguin 12/362 unambiguously identify it as an apicomplexan. However, these features are broadly characteristic of the phylum, and further identification to the level of genus, or even family, is not possible on this evidence. The most likely pathogenic apicomplexans to consider are the

haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*), the piroplasmid *Babesia* and various genera of coccidia including *Toxoplasma*, *Eimeria*, *Isospora*, *Atoxoplasma* and *Sarcocystis*[6]. The morphologic features of the disease identified by post mortem (that is, random multifocal necrosis in the liver and spleen) make *Babesia* infection relatively unlikely, given that *Babesia* is strictly an erythrocyte parasite in the vertebrate host, and it does not replicate in fixed cells in tissues[40]. With no evidence of involvement of the gastrointestinal tract, or of development to micro- and macrogamete stages, the likelihood of *Eimeria* infection is greatly reduced[6]. *Sarcocystis* is rarely pathogenic in birds, and acute pathological changes, when they do occur, tend to be most severe in the lungs[6]. Of the remaining organisms, the gross and microscopic findings are non-specific and may be compatible with an infection with any one of them.

The apicomplexans are a diverse and complex group, and their life cycles resist generalisation. However, at a very simple level, these protozoa have both sexual and asexual stages of reproduction. Sexual reproduction involves the fusion of macrogametes to microgametes by a process of syngamy[76], and by definition it occurs in the definitive host, though the macro- and microgametes themselves may have formed in either the definitive host or an intermediate host. Sexual reproduction results in the production of infective sporozoites, which, in the appropriate host tissue, multiply by endopolygeny to produce one or more generations of merozoites. At some point, merozoites divide to form macro- and microgametes for the next round of sexual reproduction. In *Toxoplasma* asexual reproduction occurs by a process of endodyogeny, a special form of endopolygeny in which only two daughter cells are produced both by incorporating some organelles from the mother cell and by de novo synthesis of others[35,60]. Endodyogeny is not unique to *Toxoplasma*, however, having been reported in the apicomplexan genera *Cytoisospora*[21], *Isospora*[55], *Sarcocystis*[66], *Besnoitia*[24] and *Neospora*[81]. Thus, the several instances identified in these photographs in which a pair of parasites is visible within a larger cell are suggestive of, but not definitive for, *Toxoplasma* infection.

Many of the protozoa seen in these images are extracellular. The asexual stages of the apicomplexan life cycle involve penetration of sporozoites or merozoites into host cells followed by reproduction in the host cell cytoplasm or in a cyst. After reproduction, the parasites lyse the host cell and emerge to the extracellular environment whereupon they may infect new cells. Thus, a heavy parasite burden can cause significant host tissue necrosis, which, if widespread, or if multiple tissues are involved, can induce a systemic inflammatory response. While the parasites in the images above are often well-preserved, the background cells are usually degenerate, which is

consistent with the light microscopic findings and the hypothesis that the penguins ultimately succumbed to a state of shock mediated by cytokine overproduction.

In conclusion, the infection in the penguins is confirmed to be due to an apicomplexan parasite. Further identification on morphology alone is not expected to be definitive. Molecular identification by techniques such as the Polymerase Chain Reaction (PCR) are required.

5. Molecular Identification of the Parasite

Introduction and Aims

From the light microscopy and electron microscopy investigations to this point, the morphological characteristics of the parasite in the penguin tissues allow it to be identified as a protozoan parasite in the phylum Apicomplexa. However, definitive identification at least to the level of genus is necessary to allow an appropriate understanding of the disease. The most definitive means by which this may be achieved is amplification and sequencing of the organism's DNA using the Polymerase Chain Reaction (PCR) technique. PCR is a process whereby oligonucleotide primers are used to induce the targeted replication of segments of DNA in large numbers, permitting that DNA then to be dyed and visualised as distinctive bands on an agarose gel. The amplified segments of DNA can be collected, the nucleotide sequence can be determined, and analysis of that sequence allows a comparison to be made with a database of known DNA sequences that are characteristic of specific organisms.

With respect to the penguins, samples of multiple tissues, usually including liver, spleen, kidney, heart, brain and adipose tissue, have for some years been routinely collected and stored at -20°C for all penguin post mortems conducted at Murdoch University, primarily for the purposes of toxicological assays. Thus, tissue samples were available from this archive allowing DNA extraction for PCR. In principle, to identify the agent causing the deaths of these penguins, it should be demonstrable that DNA from a single parasite is present in all of those birds diagnosed with protozoal infections. Thus, the aims of PCR in this context were:

1. To prove that an apicomplexan parasite can be detected and identified in the affected tissues
2. To identify the parasite to at least the level of genus
3. To validate an in-house PCR that can be carried out at Murdoch University on these and future cases for definitive diagnosis

All case numbers referred to in this section are as presented in Table 1 in chapter 2. Prior to undertaking PCR for this project, tissue samples from 10 cases (cases 1, 2, and 4 to 11 inclusive) had been submitted to an external laboratory (Center for Evolutionary and Theoretical Immunology, University of New Mexico, Albuquerque, NM, USA) for PCR assessment with the intention to demonstrate *Plasmodium* and/or *Haemoproteus* cytochrome-b DNA. From these ten samples, positive findings were obtained from four: cases 5, 8, 9 and 10[14]. The PCR products from each of

these positive cases were sequenced, and they were identified as representing a novel species of *Haemoproteus* in the subgenus *Parahaemoproteus*. Samples from these cases were included in the current PCR investigation described in this thesis, and the four positive samples were considered to represent positive controls for *Haemoproteus*. The PCR protocol used in this project was based on that published in Cannell *et al*, 2013[14]. Consequently, a supplementary aim was derived from this publication, namely:

4. To verify the results from the external laboratory that found *Haemoproteus* in the penguin tissues

In addition to testing for *Haemoproteus*, the penguin DNA was also used in PCR assays with primers designed to detect piroplasmids, apicomplexans generally, and *Toxoplasma*. The reasons for running these assays are discussed more fully in chapter six of this thesis; in essence, however, they were performed to broaden the investigation as a consequence of the consistently negative results obtained from the *Haemoproteus* assays (described below).

Materials and Methods

DNA extraction

DNA was extracted from a frozen sample of liver when available, or from spleen when liver was not kept. Samples were collected from 15 birds, all of them with microscopic evidence of protozoan parasite infection. The 15 cases were: 1 to 10 and 12 to 16. Nine of these (1, 2 and 4 to 10) were from the same cases that had been tested in New Mexico; one case (case 11) was excluded as no archived frozen tissue could be found for this bird. Six additional samples were included as they had been received for post mortem after the previous group of samples had been submitted to New Mexico (cases 12 to 16), or, in one case, because the diagnosis of protozoan infection was not made until a later review of cases (case 3).

DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's instructions. Briefly, for each sample approximately 25mg of tissue was chopped finely with a fresh scalpel blade and collected into a 1.5mL microcentrifuge tube. After the addition of 20µL of proteinase K, the tube was briefly vortexed then incubated at 56°C for 3 hours. The samples were vortexed again after removal from the incubator, and then 200µL of lysis buffer (Buffer AL) were added. After vortexing again, 200µL of 100% ethanol were added before a final vortexing step. The mixture from this tube was aspirated into a spin column placed into a collection tube. The spin

column was centrifuged at 6000g (8000rpm) for 1 minute. The spin column was then transferred to a new collection tube and 500µL of wash buffer (Buffer AW1) was added before a second centrifugation step at 6000g. Again, the spin column was transferred to a new collection tube and 500µL of a second wash buffer (Buffer AW2) was added before a final 1 minute centrifugation at 20000g (14000rpm) to dry the DNA binding membrane. The spin column was placed in a clean 1.5mL microcentrifuge tube and 200µL of elution buffer (Buffer AE) was pipetted onto the spin column membrane. This was allowed to incubate for 1 minute at room temperature, and then the sample was eluted by centrifugation for 1 minute at 6000g. A second, identical elution step was performed to maximise the DNA yield. The above protocol was followed for each tissue sample, and also for an extraction blank negative control, which contained no tissue. After extraction, the samples were stored at -20°C until required.

Prior to amplification, the samples from the first extraction were analysed for DNA yield and purity by spectrophotometry. The DNA concentration for each tube was estimated by calculation from the absorbance of the solution at a wavelength of 260nm. The purity of the sample was estimated by the ratio of absorbance at 260nm (mostly DNA) to that at 280nm (mostly proteins). The DNA yield and A_{260}:A_{280} values are presented in Table 1. The DNA yield and purity in each case were considered acceptable. This analysis was not performed on the samples from the second extraction.

Case No.	DNA (ng/µL)	A_{260}:A_{280}
1 – 06/1172	521.6	1.59
2 – 08/1075	309.2	1.20
3 – 11/484	332.4	1.57
4 – 11/624	211.6	1.28
5 – 11/626	155.2	1.69
6 – 11/627	242.2	1.30
7 – 11/628	352.0	1.71
8 – 12/020	323.8	1.38
9 – 12/021	298.8	1.24
10 – 12/338	356.2	1.39
12 – 12/375	368.9	1.42
13 – 12/385	413.8	2.04
14 – 12/473	326.2	1.94
15 – 12/517	555.2	1.38
16 – 12/524	461.1	1.27
negative control	1.7	1.59

Table 1 – DNA yield and purity

A second DNA extraction was performed after the first run of *Toxoplasma* PCR (discussed below), as the extraction blank negative control returned a positive result, indicating that there had possibly been contamination of this tube during the extraction process. For the second extraction, a further eight samples were included as negative controls from penguins which did not have gross or microscopic post mortem findings suggestive of protozoal infection. The case numbers and post mortem diagnoses for these additional penguins are presented in Table 2. The second DNA extraction also included those cases listed in the first DNA extraction, with the exception of case 10; there was a labelling error during the extraction process with the result that this sample could not be confidently identified, so it was discarded. The protocol for the second DNA extraction was identical to the first, though the quantity of tissue from which the extraction was made was, in each case, reduced to approximately 5mg. This adjustment was made on the advice of a colleague experienced in the process, with the rationale that lysis of the tissue would be more effective, reducing residual particulate matter in the final solution without compromising the yield of DNA.

Case Number	Post mortem diagnosis
NC1 – 11/080	Proventriculitis with *Contracaecum* sp. infection
NC2 – 11/452	Inconclusive findings
NC3 - 11/482	Starvation
NC4 - 11/625	Trauma
NC5 - 12/015	Inconclusive findings
NC6 - 12/016	Inconclusive findings
NC7 - 12/571	Inconclusive findings/starvation
NC8 - 13/305	Starvation

Table 2 – additional (negative control) samples, negative by light microscopy for protozoan parasites

DNA from the first extraction was used in Haemosporidian and piroplasmid rounds 1 to 4 and in *Toxoplasma* round 1 detailed below.

DNA from the second extraction was used in the Haemosporidian PCR round number 5 and in the *Toxoplasma* PCR round number 2 detailed below.

Polymerase Chain Reaction

Haemosporidian and piroplasmid parasites

Several rounds of PCR were conducted using primers designed to detect various genera of apicomplexan protozoa. These rounds are presented in temporal sequence.

For the haemosporidian parasites, primers for nested PCR were supplied by Invitrogen/Life Technologies using the sequences published in Cannell *et al*, 2013 [14], which had been designed to detect haemosporidian parasites. The primer sequences for the primary PCR, designated HaemNF (forward) and HaemNR2 (reverse), were CAT ATA TTA AGA GAA TTA TGG AG, and AGA GGT GTA GCA TAT CTA TCT AC respectively (note: all sequences here and in the remainder of this chapter are presented from 5' to 3'). The primer sequences for the secondary PCR were HaemNF1 (forward) CAT ATA TTA AGA GAA ITA TGG AG, and HaemNR3 (reverse) ATA GAA AGA TAA GAA ATA CCA TTC. The target of these sequences was a fragment of the mitochondrial cytochrome-b gene which is common to *Plasmodium* and *Haemoproteus*, with an expected product of approximately 480 base pairs in length[14].

For piroplasmid parasites, the primer sequences for the primary PCR, designated BTF1 (forward) and BTR1 (reverse), were GGC TCA TTA CAA CAG TTA TAG, and CCC AAA GAC TTT GAT TTC TCT C respectively. The primer sequences for the secondary PCR were BTF2 (forward) CCG TGC TAA TTG TAG GGC TAA TAC, and BTR2 (reverse) GGA CTA CGA CGG TAT CTG ATC G. These sequences are as published in Jefferies et al, 2007[44].

For *Toxoplasma* genus parasites, the primer sequences for the primary PCR were TGT TCT GTC CTA TCG CAA CG (forward) and ACG GAT GCA GTT CCT TTC TG (reverse). For the secondary PCR, the primer sequences were TCT TCC CAG ACG TGG ATT TC (forward) and CTC GAC AAT ACG CTG CTT GA (reverse). These sequences are as published in Grigg and Boothroyd 2001[34].

PCR round 1

For the first round of PCR, 1:10 dilutions were made from each DNA sample. Diluted DNA was used initially in order to reduce the potential for enzyme inhibitors, which may be present in DNA extractions from tissues, to interfere with polymerase activity. A PCR master mix was prepared which consisted of 10 µL TAQ DNA Polymerase, 250µL of reaction buffer, 50µL of 10mM dNTP mix, 540µL of cresol red dye, 150µL of 25mM $MgCl_2$ and 250µL of purified water. PCR tubes were then prepared, which contained 12.5µL of PCR master mix, 1µL each of HaemNF and HaemNR2 primers, 9.5µL of purified water and 15.5µL of cresol red dye. One µL of dilute DNA was then added to each tube and the tubes were briefly centrifuged.

A complete set of duplicate tubes was made which were identical except for the substitution of BTF1 and BTR1 primers in place of the HaemNF and HaemNR2 primers.

The thermal cycle program for the primary PCR was as follows: the samples were brought to 94°C for 8 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 45 seconds. On completion of the 35 cycles, the samples were held at the 72°C extension temperature for a further 10 minutes, then at 4°C until removal from the thermocycler.

For the secondary PCR, a set of tubes were prepared which were identical to the primary PCR, with the substitutions of HaemNF1 and HaemNR3 primers (haemosporidian), and with 1µL of DNA taken from the primary PCR products. The secondary PCR was run with the same conditions as the

primary PCR, with the exception that the annealing temperature was 52°C rather than 50°C. A secondary PCR with the primers BTF2 and BTR2 (piroplasmid) was also run.

After thermal cycling, 15-18µL of the PCR products were pipetted into the wells of an agarose gel (1% agarose in Tris-acetate-EDTA buffer containing SYBR Safe DNA gel stain), across which an electrical potential of 76 volts was applied for 40 minutes. On completion, the gels were immediately photographed under ultraviolet light to detect the presence of bands at approximately 480bp (haemosporidians) or 360bp (piroplasms). Faint bands were detected for cases 5 and 6 on the haemosporidian gels. The positive bands were excised from the gel and the DNA was collected by centrifuging the bands at 10000g for 5 minutes through a filtered pipette tip into a 1.7mL micro tube.

PCR round 2

A second round of PCR was performed using undiluted DNA from the same cases and a freshly prepared batch of master mix (Taq polymerase, reaction buffer, dNTPs, cresol red, $MgCl_2$ and purified water). The protocol was otherwise unchanged.

PCR round 3

A third round of PCR was performed using DNA from the PCR products of the second round. The products of those cases that had produced strong bands in the round 2 *Haemoproteus* PCR (see below) were used as the samples for this round of *Haemoproteus* PCR. The products of those cases that had produced bands in the round 2 piroplasmid PCR were used as the samples for this round of piroplasmid PCR. The PCR products were considered to represent amplified DNA; as such the primary PCR step was omitted from the protocol and only the secondary PCR was performed.

PCR round 4

A fourth round of PCR was performed using undiluted DNA from all cases. In this round, two sets of previously unused primers were used. The first set of primers had been newly developed to detect the 18S ribosomal subunit from piroplasms; the primer sequences were F4 (forward) ACG AAC GAG ACC TTA ACC TGC TA and R7 (reverse) GGA TCA CTC GAT CGG TAG GAG. The second set of primers were generic apicomplexan primers which amplify the 18S rRNA gene as published by Yang et al, 2014[88]; the primer sequences were Crypto F0 (forward) AAC CTG GTT GAT CCT GCC AGT and Crypto R0 (reverse) GCT TGA TCC TTC TGC AGG TTC ACC TAC.

PCR round 5

In July 2014, a corrigendum was published in Veterinary Parasitology, indicating that, due to a transcribing error, the primer sequences that had been published for the secondary PCR were incorrect[13]. In consequence of this, a new set of primers was obtained. The new sequences for the secondary primers were HaemF (forward) ATG GTG CTT TCG ATA TAT GCA TG and HaemR2 (reverse) GCA TTA TCT GGA TGT GAT AAT GGT; the sequences of the primary PCR primers were unchanged.

Toxoplasma

PCR rounds 1 and 2

Toxoplasma PCR was performed by Adriana Botero-Gomez, PhD candidate in parasitology at Murdoch University. PCR tubes were prepared containing 2.5µL of buffer, 1.5µL of $MgCl_2$ (25mM), 1µL of dNTPs, 1µL of each primer (obtained from Invitrogen/Life Technologies), 0.2µL of Taq polymerase, 8.8µL of water and 6µL of Cresol. One µL of DNA was added to each tube to give a total reaction volume of 25µL.

The thermal cycle program for both the primary and secondary PCRs was as follows: the tubes were held at 95°C for 1 minute, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute. At completion of these cycles the samples were held at 72°C for a further 5 minutes, then at 10°C until removal from the thermocycler.

DNA sequencing

Prior to sequencing in each case, a sequencing reaction was performed to amplify the PCR products. In this reaction, PCR tubes were prepared using 6.5µL of DNA, 1.5µL of 5x buffer, 0.7µL of dye and 1.3µL of 3.2µM forward primer. The tubes were placed in a thermocycler using the same program as that described for the secondary PCR above.

On completion of the sequencing reaction, the DNA product was precipitated in preparation for sequencing. To precipitate the DNA, 10µL of the PCR products were added to the wells of a 96 well plate, to which were added (in order) 1µL of 125mM EDTA, 1µL of sodium acetate (3M, pH 4.6) and 25µL of 100% ethanol. The plate was shielded from light and incubated for 20 minutes at room temperature. After incubation, the plate was centrifuged at 4060g for 35 minutes, then it was inverted and pulse centrifuged to 185g. 35µL of 70% ethanol were added to each well, and the plate was centrifuged at 1650g for 15 minutes after which it was inverted again and pulsed to 150g. The plate was allowed to air dry for 5 minutes, wrapped in alfoil to protect it from light and then

submitted to the State Agricultural Biotechnology Centre located on the Murdoch University South Street campus for sequencing. Sequencing was performed on an ABI 3730 96 capillary sequencer by the technical staff of that laboratory.

Results

Haemosporidian and piroplasm parasites

PCR round 1

Haemoproteus PCR - faint bands were detected in the gel at approximately 480bp for cases 5 and 6, indicating potential positive results. These bands were excised and the DNA collected by centrifugation as described. On sequencing, the DNA was found to be of poor quality, both sequences were different to one another, and neither was specific for any class of organism.
Piroplasm PCR – no bands indicative of piroplasm DNA were detected.

PCR round 2

Haemoproteus PCR – strong bands were detected for cases 1 to 8. These cases were in lanes 2 to 9 on the gel, lane 1 being the DNA ladder. The remaining cases were at the base of the same gel, and in these cases no bands were seen. However, the corresponding DNA ladder extended beyond the edge of the gel. Consequently, bands in these lanes may have been undetected either because they were truly absent, or because they had run off of the edge of the gel. All positive bands were excised, processed as described and submitted for sequencing. For all of these samples, the DNA was of poor quality, the sequences generated were non-specific, and all sequences were non-identical.

Piroplasm PCR - bands of DNA were observed in cases 1 and 3. These bands were excised, processed as described above and submitted for sequencing. For both of these samples, the DNA was of poor quality, the sequences were non-specific, and both sequences were non-identical.

PCR round 3

In the third round of PCR, no bands were detected in any of the *Haemoproteus* PCR samples. Strong bands were detected in cases 1 and 3 from the piroplasm PCR. These 2 bands were excised, processed as described and submitted for sequencing. The sequences were of poor quality, the sequences generated were non-specific, and both sequences were non-identical.

PCR round 4

Piroplasm PCR – bands of DNA were observed in all cases. These bands were excised, processed as described above and submitted for sequencing. A valid sequence could not be generated for case 12. All other cases returned valid sequences, which were submitted to a search on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). From this search, the PCR products from all samples were identified as belonging to the family Sarcocystidae. Resolution to the level of genus was not possible.

Apicomplexan PCR – bands of DNA were observed in 11 cases (1 to 7, 10, and 14 to 16). These bands were excised, processed as described above and three were submitted for sequencing (1, 2, and 10). A valid sequence could not be generated for case 1. Sequences for cases 2 and 10 were generated; the quality of these sequences was good for approximately the first 300 base pairs, but deteriorated and became unreliable after that (NB: the PCR products from these primers are very long, about 1584 bp). These sequences were consistent with, but not definitive for, *Toxoplasma*.

PCR round 5

In the fifth round of PCR, using the corrected primers for *Haemoproteus*, the samples tested were from the second DNA extraction described above. The protocol followed was that published in the corrigendum (unchanged from the original paper other than the altered primer sequences), and a single faint band was detected in the lane of sample 15. The band was cut from the gel and processed for sequencing. However, the sample failed to produce a valid sequence.

Toxoplasma

PCR round 1

Each sample was strongly positive for *Toxoplasma*, though this included the negative control. The result for the negative control indicated that there had been contamination of the samples either during DNA extraction or during the PCR process. Consequently, it could not be determined which samples were true positives and which were false positives.

PCR round 2

In the second round of PCR, the samples tested were from the second DNA extraction described above. Fifteen samples were positive and six were negative (see Table 2 and Figures 1 and 2). All DNA extraction blank and PCR negative controls were negative.

Case No.	Parasites detected by light microscope	Toxoplasma detected by PCR
1 - 06/1172	+	+
2 - 08/1075	+	+
3 - 11/484	+	+
4 - 11/624	+	+
5 - 11/626	+	+
6 - 11/627	+	+
7 - 11/628	+	+
8 - 12/020	+	+
9 - 12/021	+	-
10 - 12/338	+	+
12 - 12/375	+	+
14 - 12/473	+	+
15 - 12/517	+	+
NC1 - 11/080	-	-
NC2 - 11/452	-	-
NC3 - 11/482	-	-
NC4 - 11/625	-	+
NC5 - 12/015	-	-
NC6 - 12/016	-	+
NC7 - 12/571	-	+
NC8 - 13/305	-	-

Table 3 – Light microscopy and *Toxoplasma* PCR results

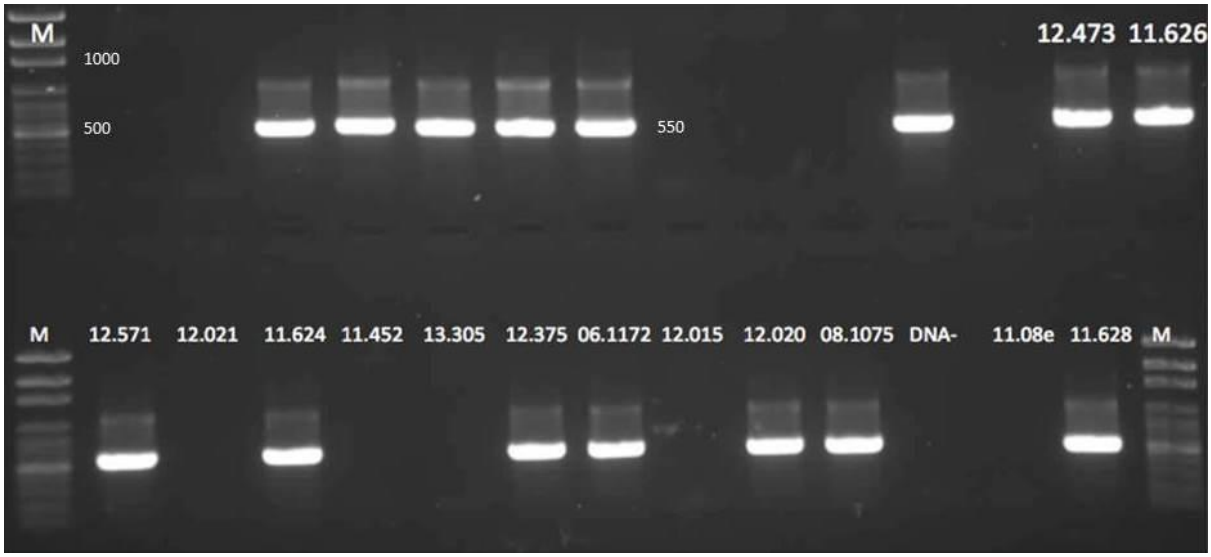


Figure 1 – *Toxoplasma* PCR gel #1 (ladder and amplicon sizes indicated in base pairs)

Figure 2 – *Toxoplasma* PCR gel #2 (ladder and amplicon sizes indicated in base pairs)

Amplicons from 15 of the penguin samples were sequenced. As only a single locus (B1) was amplified, it is possible to say that the samples are either Archetype II or III; to distinguish between them, an additional locus would need to be amplified and sequenced as well (Adriana Botero-Gomez, pers comm.)

Immunohistochemistry

Immunohistochemistry (IHC) is a histological technique that uses labelled antibodies to identify specific antigens in situ on slide-mounted tissue sections. The range of antigens that can be identified in this way is effectively limited to those for which a commercial antibody is available. For the present study, IHC was accessible to detect *Toxoplasma*, but not for *Haemoproteus*.
IHC was performed on 6 samples that had routine histological evidence of protozoan infection, specifically on cases 2, 3, 9, 10, 14 and 15. Two additional samples with no histological or PCR evidence of protozoan infection were also included, cases NC1 and NC2 from table 2 (negative controls).

Toxoplasma IHC was performed by the technical staff of the Murdoch University Veterinary Hospital histology laboratory (School of Veterinary and Life Sciences) using a polyclonal antibody (Thermo Scientific Polyclonal (Goat) PA5-16921). Polyclonal antibody was used because it detects multiple life stages of toxoplasma, and it is thus more sensitive than monoclonal antibody, but without a loss of specificity; the antibody does not even cross react with the closely related *Neospora caninum*, so its specificity is considered to be excellent (Michael Slaven, senior histology technician, MUVH, pers comm.) Briefly, the protocol was as follows: each slide was washed in water, then treated with 3% Hydrogen Peroxide for 15 minutes. Slides were washed with distilled water followed by TRIS wash solution, and then treated with normal goat serum for 10 minutes. The goat serum was poured off and the antibody applied and then incubated for 45 minutes. Negative control slides were prepared omitting the antibody application, but washing with the same buffer solution. All slides were then washed and treated with the secondary antibody reagent for 30 minutes, after which they were washed and treated with a diaminobenzidine substrate-chromogen solution for 3 minutes. Slides were counterstained with Harris' Haematoxylin, washed in Scott's tap water substitute, then dehydrated, cleared and coverslipped with DPX.

Results

In each of the cases that had routine light microscopic evidence of tissue necrosis and protozoan parasites, there was distinct positive staining, evident at low and high power, which was colocalised with the necrotic regions (Fig 3). In the cases without evidence of parasites, there was no additional staining beyond the background stain seen in the negative control slides.

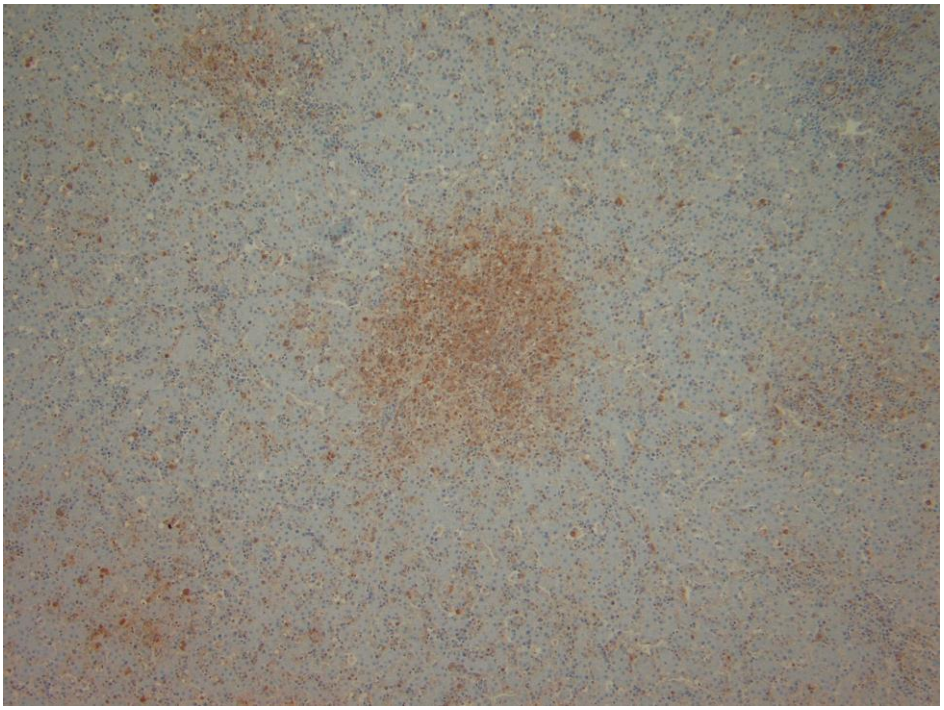


Figure 3 – *Toxoplasma* polyclonal antibody IHC. Several foci of brown staining, most notably in the image centre, indicate *Toxoplasma* antigen within a necrotic focus, liver, case 15, x100 magnification

At high power, the uptake of stain was clearly associated with the organisms in the lesions (Fig 4).

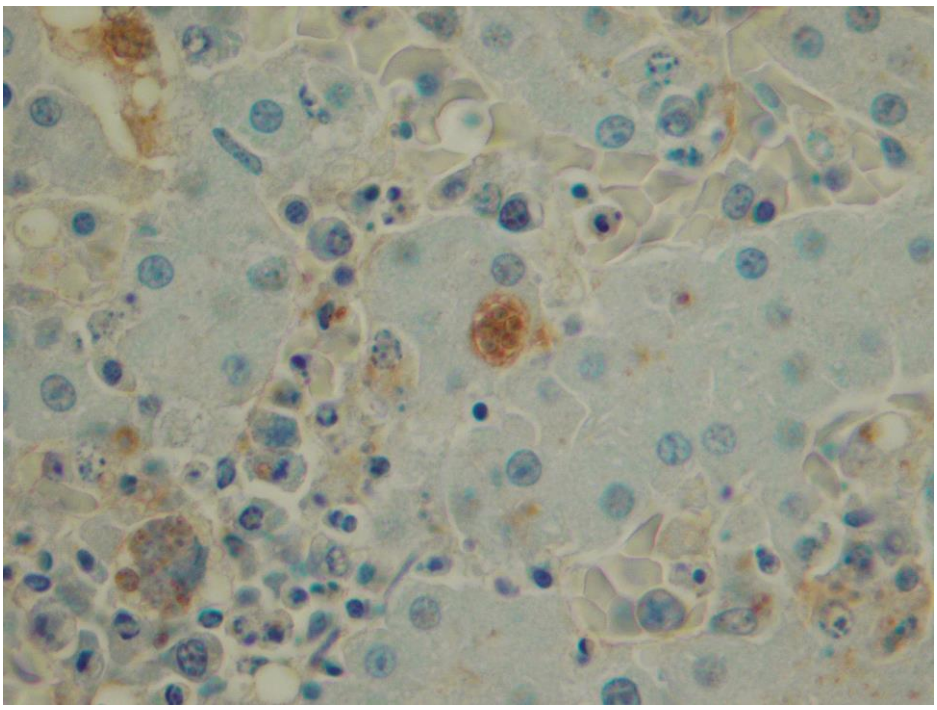


Figure 4- *Toxoplasma* polyclonal antibody IHC. Brown staining of several protozoan cysts within a hepatocyte, liver, case 15, x1000 magnification

Discussion

Although many of the same tissue samples were used for DNA extraction, including those which had tested positive for *Haemoproteus*, the results of the haemosporidian PCR as published by Cannell *et al*, 2013 [14] could not be reproduced in the first attempts at PCR. This was an unexpected result given that the assays had been performed using the same primer sequences and the same protocols. However, in that paper it was noted that only 4 of 10 penguins were positive by PCR, even though all 10 had histologically identifiable parasites in liver and spleen sections. Also, of those that were positive, the PCR protocol had been repeated on up to 3 occasions before DNA was detected, indicating a lack of sensitivity for this assay. Possible reasons for the poor sensitivity include: a) a low concentration of DNA in the samples, b) interference from a reaction inhibitor, or c) degradation of reaction reagents. Perhaps significantly, it has previously been reported that nested PCR is in fact not a sensitive technique for the detection of mixed haemosporidian parasite infections in birds[84]. Consequently, and particularly given that we did not have access to a dependable positive control for these assays (notwithstanding that 4 of the samples had tested positive in another lab), the failure to detect *Haemoproteus* DNA could not be confidently interpreted as indicating that the birds were clear of this parasite.

In the present study, for the first two rounds of PCR using the published haemosporidian primers, there were numerous instances in which a band was detected on the gel; however, the DNA sequences were invariably of poor quality with multiple signals at many of the data points. It was presumed that these bands had arisen from non-specific DNA amplification due to aberrant binding of the primers to host or contaminant DNA. However, toward the end of the time and funding allocated to this project, the failure to detect *Haemoproteus* DNA (and possibly the poor quality of the bands that were produced) was explained, at least in part, by the revelation that the primer sequences that had been published were incorrect. New primers were obtained to attempt the PCR, though with very limited time and remaining funds available only one round of PCR was possible. From this assay there was a single sample, from case 15, for which a band was detected, though again no valid sequence could be generated from this sample. Of the four samples that had tested positive in the Cannell *et al*, 2013 paper (that is, cases 5, 8, 9 and 10), none were positive, though case 10 was in fact not included in the final assay as the sample had been mislabelled. In summary, the *Haemoproteus* PCR results in this study did not in any way clarify the nature of the infection affecting these birds.

On the other hand, the identification of Sarcocystidae family DNA was unexpected, and was essentially fortuitous. The penguin samples had been included in a mixed batch of samples from multiple sources as part of the validation process for a set of newly developed primers designed to detect piroplasmids. Given that they had previously tested as negative for piroplasmids, they were expected to be negative again. The positive result from this PCR was the most forthright indication to this point that apicomplexans from another family than the Haemosporida might be implicated in the disease process in the penguins (note that this result also indicates that the new primers were less specific for piroplasmids than the developer had anticipated). Probably the most noteworthy member of the Sarcocystidae, certainly in terms of its pathogenicity, is *Toxoplasma gondii*, and it is notable that fatal toxoplasmosis has been reported in a Little Penguin[56] with post mortem findings similar to those observed in these cases. The suggestion arising from this PCR, and also from the generic apicomplexan PCR, that the organism might be *Toxoplasma* led to a more directed effort to demonstrate that organism.

The results of the first round of *Toxoplasma* PCR, while uniformly positive, were discarded on the basis of a positive result from the negative control indicating contamination of the samples. However, after a second DNA extraction with appropriate control outcomes, *Toxoplasma* was detected by PCR in 12 of 13 penguins diagnosed with protozoal infections, and in 3 of 8 penguins diagnosed with other causes of death (see Table 3) (Note also that the penguin negative for *Toxoplasma* by PCR was positive by IHC – see further discussion of this finding below). Using a web-based relative risk calculator (http://www.medcalc.org/calc/relative_risk.php, accessed 17th September, 2014), the relative risk of death with evidence of protozoal infection for the penguins positive for *Toxoplasma* by PCR is 4.8 with a 95% confidence interval of 0.7879 to 29.2432. However, the p-value for this calculation is 0.0889, so it is not statistically significant using the conventional cut off for significance of 0.05. Increased numbers of penguins in these groups, especially the group without histologically detectable parasites, are required to give a more statistically reliable result.

On the sum of the molecular evidence available, it is likely that some of the penguins were, or had been, infected with *Haemoproteus* parasites. Given the apparent insensitivity of the PCR for this organism, it cannot be stated with confidence how many birds were truly infected. In general, as has been discussed in Chapter 1, *Haemoproteus* infections in birds are quite common and usually cause mild or no clinical disease, though there are exceptions to this observation, especially when

infection occurs in aberrant hosts. Three hypotheses may be advanced to account for the *Haemoproteus* findings:

1. The penguins are adapted hosts for the described species of *Haemoproteus,* and the few positive cases are essentially incidental
2. The penguins are aberrant hosts newly exposed, the *Haemoproteus* parasite is responsible for the severe disease and death observed, and it is only the insensitivity of the PCR that obscures a clearer relationship between the two
3. *Haemoproteus* as a co-infection is able to exacerbate another disease state, in this instance toxoplasmosis, resulting in the observed severe disease and death, but it is neither sufficient nor necessary for expression of the disease

On the evidence at hand, the contribution of *Haemoproteus* to the observed pathology is effectively unknown.

The presence of *Toxoplasma*, however, is strongly correlated with disease in these birds, and immunohistochemistry positively identifies the parasites within the lesions of 6 out of 6 of the affected birds; note that one of these (case 9 - 12/021) was the single case that was positive by light microscopy but negative by PCR, suggesting that the PCR result may represent a false negative. Given that the sample was run together with all of the other positive samples, it is clear that the reagents were in good condition and there were no concerns with the presence of inhibitors, so a false negative cannot be due to those factors. Most likely, the negative result can be attributed to an error during the extraction process, or to low DNA concentration in the sample; a repeat extraction and PCR with appropriate controls would be necessary to investigate this further. Death from toxoplasmosis has been reported in one Little Penguin previously, with similar post mortem findings to those in this study[56]. The gross, histological, haematological, ultrastructural, immunohistochemical and PCR results are all consistent with fulminant toxoplasmosis being the cause of death in these penguins.

6. Discussion

An investigation of the cause (or causes) of a naturally occurring outbreak of disease starts with observations of the disease and its manifestations, followed by the generation of a hypothesis as to the aetiology, and then testing of that hypothesis. The first instances of the disease explored in this thesis were two isolated cases separated by two years. Under these circumstances, the threat of the disease to the wellbeing of the penguin population appeared to be minimal, so a provisional diagnosis was acceptable in each case, and there was little to no urgency to follow up with additional tests to establish a definitive diagnosis. The sudden increase in cases that was observed in late 2011 and early 2012, especially in light of the recent history of declining numbers of breeding penguins on Penguin Island, meant that a definitive diagnosis came to be considered important. Understanding the aetiology is the first step in developing a strategic response, or in determining whether a response is feasible, so the aims of this project were:

1. To document the post mortem findings that are associated with this protozoan infection
2. To identify the parasite at least to the level of genus, and
3. To commence collection of data intended to assess the prevalence of the disease in the live penguin population and in potential avian hosts other than the penguins

The provisional diagnosis of Avian malaria, which is to say, infection with one or another species of *Plasmodium*, was reasonable given the gross and histological findings at post mortem, and the knowledge that Avian malaria is a significant cause of morbidity and mortality in penguins, though the link between infection and disease has been better documented in birds in captivity than in those in the wild. Thus, the hypothesis for this investigation (with respect to the identity of the parasite) was that those penguins presenting with the distinctive signs of multifocal hepatic and splenic necrosis with enlargement of those organs had been infected with *Plasmodium* parasites. The very low (near to non-existent) incidence of parasitaemia, as judged from histological assessment of erythrocytes and occasionally from cytology of tissue impression smears, was argued to be because the animals presenting for post mortem had died in the acute phase of infection from extensive tissue necrosis leading to the development of a cytokine-mediated haemodynamic shock. In terms of pathogenesis, this explanation was, and remains, reasonable. In fact, the pathological interpretation of the lesions has not changed since the project was undertaken – the only real unknown was the identity of the protozoa in those lesions.

The earliest work done in the project was the collection of blood samples from Little Penguins and Bridled Terns to establish whether parasitaemia could be detected in either of these species, and, if it could, to estimate the prevalence of infection. An earlier haematological study published in 1999 did not detect parasitaemia in any penguins from this population[46]. However, given that we suspected that the infection was relatively new to these birds (on the basis that it was causing multiple mortalities, as would be expected in an immunologically naïve population) our assumption was that overt parasitaemia would now be more common. From the 146 blood smears examined (94 Little Penguins, 52 Bridled Terns), blood parasites were found in the erythrocytes of one penguin only. On morphological grounds, this parasite could be identified as an apicomplexan, but not further. Assuming that this parasite was the same as that causing the fatalities (which might not be the case) this evidence suggested that the prevalence of infection was most likely low. Also, since the bird in question was not obviously unwell, and that there was evidence of a regenerative red cell response and the parasitaemia was only mild, it appeared that the infection was at least not uniformly fatal.

Subsequent to the blood sampling, transmission electron microscopy was performed using tissue from one penguin, and from this it was possible to identify characteristic features of apicomplexan protozoa in the splenic tissue. While this confirmed the light microscopic findings to a much higher resolution, few specific features that might further narrow down its taxonomy were found. The one feature that was somewhat helpful was that the parasites appeared to multiply by a process of endodyogeny, though even this feature has been documented in many apicomplexan genera (not, however, including *Plasmodium*).

Thus, in terms of testing the hypothesis, early investigations could not demonstrate that the parasite was *Plasmodium*. At around this time, a PCR investigation conducted by an external laboratory offered the explanation that the parasite was in fact from the genus *Haemoproteu*s, an organism which is so closely related to *Plasmodium* that it was detected by PCR primers that were originally designed to identify *Plasmodium*. Diagnostically, the similarities between the two parasites are such that, where morphology of the erythrocyte stages of development are lacking (*Plasmodium* spp. undergo shizogony in erythrocytes whereas *Haemoproteus* spp. do not), they can only be distinguished by techniques such as DNA sequencing. In terms of pathogenicity, also, the two parasites can be similar, differing primarily in features such as host range and vector; indeed, some reports of Avian malaria group *Plasmodium* and *Haemoproteus* infections together, though it is more usual to consider them as distinct disease entities. Consequently, the focus of the project

shifted to investigating the hypothesis that this fatal disease was haemoproteosis rather than Avian malaria.

However, the evidence to implicate *Haemoproteus* as the infective agent was not without its own problems. To start with, only 4 of the 10 cases assayed were positive by PCR, though all had clear and consistent evidence of morphologically identical parasites in the tissues by routine light microscopy. Additionally, even among the cases that were positive by PCR, they were not positive all of the time. There was a distinct lack of sensitivity to the assay (assuming that the parasite in each of the cases was the same) that made confident interpretation difficult, particularly in regard to those samples that tested negative. The fact that the DNA products had been sequenced to demonstrate a previously undescribed species of *Haemoproteus*, however, made it unlikely that there had been contamination of the penguin samples from another source, so it had to be assumed that the infection was real. The true prevalence and significance, however, were uncertain.

The picture was not clarified when I tried to replicate the PCR results in our own laboratory without success. After several attempts at the assay, a colleague assisting the investigation (Dr Andrea Paparini, Vector and Waterborne Pathogens Research Group, Murdoch University) fortuitously included the penguin DNA samples when testing some new PCR primers he had developed for working with the piroplasms *Babesia* and *Theileria*, the penguin samples in this context being intended to function as negative controls as they had already tested as negative for those organisms in an earlier PCR. Unexpectedly, this PCR generated bands of DNA, and a selection of those bands were sequenced to reveal that the organisms present were members of the family Sarcocystidae. The Sarcocystidae belong within the phylum Apicomplexa, dividing from the taxonomic branches which include *Plasmodium*, *Haemoproteus*, *Babesia* and *Theileria* at the level of class (Conoidasida versus Aconoidasida). At the same time as this assay, another PCR of the penguin DNA was performed using generic primers for apicomplexans. The PCR product for this assay is very large, and while the two samples tested were positive, a complete sequence could not be generated for either sample. However, the segment of each that did return a good sequence gave a result that was most consistent with *Toxoplasma*. *Toxoplasma* is a single-species genus (*T. gondii* is the only member) in the family Sarcocystidae, and it is a significant pathogen of wildlife, domestic animals and humans worldwide.

On the basis of these results, new PCR assays were undertaken which were directed specifically at identifying *Toxoplasma*. Immediately, every sample tested was strongly positive for *Toxoplasma*.

Unfortunately, the extraction blank negative control (the sample made at the time of DNA extraction which contained no penguin tissue) was also strongly positive, indicating that there had been contamination of this tube, and possibly other tubes, at some point in the DNA extraction and/or PCR process. A second DNA extraction was performed, this time including eight samples from penguins that did not have microscopic evidence of protozoan infections. The *Toxoplasma* PCR was repeated and 12 of the 13 samples with light microscopic evidence of protozoa were also positive by PCR; the reason for the one negative result is unknown, though repeating the assay to determine whether the result is consistent would be a worthwhile first step. The negative controls were also negative.

At around this time, we received notification that the *Haemoproteus* PCR primers we had been using in our first attempts to reproduce the *Haemoproteus* results were based on incorrect published sequences. With very limited time and resources available, we ordered new primers and repeated the *Haemoproteus* PCR once only. A single, very faintly positive band of DNA was found, this being from a new case that had not been submitted to the New Mexico laboratory. The band was submitted for sequencing, but no sequence could be generated, possibly reflecting poor quality DNA or, alternatively, degraded reagents. In the end, no confirmed positives were ever achieved from the *Haemoproteus* PCR assays performed at Murdoch University.

Perhaps belatedly, a selection of cases, both positive and negative by histology and PCR, were then investigated by immunohistochemistry, by this time with the primary intention of showing where in the tissues the *Toxoplasma* antigen was located. In all positive cases, *Toxoplasma* was demonstrated in the necrotic lesions of liver and spleen and direct staining of the protozoan cysts was apparent when the slides were assessed at high power.

The most compelling interpretation of the gross and microscopic pathology, the ultrastructure of the protozoan organism and the PCR and immunohistochemistry findings is that the penguins had been dying of acute toxoplasmosis. The pathological changes observed are in agreement with a previous published case of toxoplasmosis in one Little Penguin[56], and are also consistent with the pathology reported in experimental toxoplasmosis in other animal species (such as eastern barred bandicoots[7]) though it is worth noting that *Toxoplasma* tachyzoites and bradyzoites are widely distributed in body tissues, and the range of lesions which may occur when infections progress beyond the acute stage and become chronic is extensive[10]. *Toxoplasma* is a global parasite, which, it is emerging, is a very common infection in Australian wildlife species[65,67,68,86], and which causes fatal infections in wild

birds[23,30,41,53]. In distinction to the life cycle of apicomplexan haemoparasites, *Toxoplasma* requires no arthropod vector. The definitive host is the cat, and transmission of disease may occur by ingestion of infective oocysts from cat faeces, or by ingestion of tissue cysts in animal tissues (as well as, in all likelihood, by vertical transmission in utero[82].) Because sexual development only occurs in cats and it is not transmitted by a vector, it might be expected that exposure to *Toxoplasma* would be low in marine wildlife. However, it is also emerging that *Toxoplasma* infection is not uncommon in many marine mammals, implying that the disease can be transmitted by contamination of waterways with cat faeces[47]. Furthermore, it has been shown that filter-feeding fish will take up *Toxoplasma* oocysts from contaminated water, and that the infection can be passed to mice when fed on these fish[57]. Notably, one species of fish that has been demonstrated to take up *Toxoplasma* cysts and transmit the disease is the pilchard (*Sardinops sagax*), an important prey species for the Little Penguins of Penguin Island[57]. Also, it has recently been shown that *Toxoplasma* can aggregate on extracellular polymeric substances (EPS) in marine environments, enhancing the efficiency with which the organism is able to enter the marine food chain in coastal ecosystems[78].

Toxoplasma DNA was detected in three out of eight penguins that did not have any of the gross or microscopic lesions associated with severe protozoal disease. These birds were case number NC7, which was presumed to have died from starvation, case number NC6, for which no diagnosis could be made, and case number NC4, which was found to have died from traumatic injuries attributed to a boat strike. Each of these post mortems was conducted at or after the time of the late 2011 spike in protozoal disease, so it is unlikely that the disease was present and overlooked. The implication is that infection with *Toxoplasma* is not uniformly fatal in the penguins, and that virulence, dosage and/or comorbidity factors are likely to play a role in determining the outcome of infection in any given bird. Notably, a feature of fatal disease is that the birds were, by and large, in good body condition. It is possible that these penguins were able to gather adequate prey in the local environment without the need to travel far afield, and that the fish they were preying on in that environment themselves carried a large parasite burden, though this hypothesis would be difficult to prove. The number of penguins presented to Murdoch University for post mortem in recent years has been 26 in 2011, 40 in 2012, 22 in 2013 and none as of early October in 2014. Thus, with such low numbers to judge from, there is insufficient evidence to declare whether the cluster of cases in 2011 was a true outbreak of disease that has subsided, or whether deaths are ongoing. Whatever the case, the incidence of toxoplasmosis is most likely dependent on the both the overall number and the disease status of cats contributing to faecal contamination of local waterways. A serological

survey of the penguins would provide a useful indication of the prevalence of exposure in the population.

Ultimately, the evidence from this investigation provides no support for the original hypothesis that Avian malaria was the cause of death or disease in these penguins. Furthermore, while haemoparasite DNA has been detected in the tissues of some birds (albeit by testing at an external laboratory), the role of *Haemoproteus* in causing disease in these penguins is, unfortunately, unknown. It is likely enough that *Haemoproteus* represents an enzootic infection in these birds that had not previously been detected, at least in part because its pathogenicity was low and no effort had been made to look for it. One implication of the finding is that there is a species of ceratopogonid biting flies to which the penguins are exposed that is the definitive host for this parasite. An entomological survey of Penguin Island would be helpful in investigating this hypothesis, and demonstration of DNA from the same *Haemoproteus* parasite in any biting flies isolated from such a survey would effectively confirm it.

With regard to the other aims, the presentation of acute toxoplasmosis in Little Penguins apparently invariably includes hepatomegaly and splenomegaly with gross and microscopic evidence of multifocal areas of necrosis in these tissues. Microscopic evidence of protozoa can be found in liver and spleen sections, and occasionally in other tissues including the heart, lungs, air sacs, adipose tissue and arteries. No parasites were detected in the brain of any penguin.

Estimation of the prevalence of *Toxoplasma* infections in the Little Penguins is difficult from the evidence of this investigation, not least because blood sampling and haematology is simply an insensitive test for this purpose. A serological or PCR survey is required to answer that question. Also, given that *Toxoplasma* is a parasite of cats, the other avian species of Penguin Island can no longer be considered culpable for introducing the infection to the Little Penguins. Apologies to the Bridled Terns.

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